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Risk Management of Aflatoxin Through Mutagenic Potential Modification and Toxin Formation by Intrinsic Components in Food.

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RISK MANAGEMENT OF AFLATOXIN THROUGH MUTAGENIC POTENTIAL MODIFICATION AND TOXIN FORMATION BY INTRINSIC COMPONENTS IN FOOD

A Dissertation

Submitted to the Graduate Faculty of the
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by
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For my parents

"Wealth and Children are the adornment of this present life: but good works, which are lasting, are better in the sight of thy Lord as to recompense, and better as to hope".

(Qur'an 18:46)

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ABSTRACT

Aflatoxins are the secondary metabolites of *Aspergillus flavus* and their presence in food and feed crops is unavoidable. This study evaluates the anti-mutagenic properties of phytic and linoleic acids in aflatoxin-affected food.

A reduction in the number of revertants was a function of an increased concentration of phytic/linoleic acids and phosphotidylinositol in the *Salmonella*/microsomal mutagenicity assay (tester strains TA-100 and TA-98) with and without metabolic activation (S-9). Linoleic acid (10 and 100 μg/plate) and phosphotidylinositol (25μg/plate) were antimutagenic against most of the mutagens tested. Although a reduction in the number of revertants was observed in the phytic acid treatments, the results, however, do not suggest phytic acid as antimutagenic in this assay.

The inhibition of AFB_1 biosynthesis in the *A. flavus* inoculated Czapek-Dox liquid medium was observed as the concentration of phytic acid was increased in a 25 day study. A complete inhibition of AFB_1 production was recorded at phytic acid levels of 0.5 and 1 mg/100ml. The role of metal ions was also tested under similar conditions. In the absence of Fe^{++} and Zn^{++} ions, a complete inhibition of AFB_1 production was observed; however, Cu^{++} and Mg^{++} ions did not exhibit the same response.

In a 35 day storage study of corn the aflatoxin levels in the *A. flavus* inoculated ground and whole kernels were higher than the treatments. The reduction in the AFB_1 levels was observed after 14 days of inoculation in both the treatment and controls. Phytic acid extended the most reduction among the

treatments regardless of substrate type; however, not a complete inhibition in the biosynthesis of AFB_1 was exhibited. The production of AFB_1 and AFB_2 was significantly lower (>50%) than the *Aspergillus*-dependent and -independent controls. The production of AFB_1 in linoleic acid-treated corn was also found significantly lower (>50%) than the controls. No such observations were recorded in the combination treatment of phytic and linoleic acid. However, the production of AFB_1 was like the linoleic acid treatment.

Phytic acid and linoleic acid have been reported as antimutagenic *in vitro*. More research is warranted to evaluate their potential antimutagenic role *in vivo*.

1. INTRODUCTION

The human diet continues to depend upon cereal grains as a staple food worldwide because of their low cost and good nutritional attributes. However, such agricultural commodities are exposed to various types of infestations resulting in the production of various toxins (mycotoxins) either in the field (pre-harvest) or during their storage (post-harvest). Mycotoxins are structurally a diverse group of mostly small molecular weight compounds. They are naturally produced as secondary metabolism of fungi and their presence in the feed and food crops cannot be completely avoided. It has been estimated that 25% of the world's food crops are contaminated with mycotoxins every year (FAO, 1996). Aflatoxin B_1 (AFB_1), the most potent of mycotoxins, causes primary liver cancer (PLC) through necrosis, immune-suppression, gastrointestinal tract dysfunction, pulmonary edema, and mal-reproduction due to its teratogenicity in animals and humans. It is metabolized by the phase I enzyme system resulting in the production of highly reactive epoxides, which cause damage to cells by covalently binding to the proteins and DNA. Several reports have also documented the co-contamination of aflatoxins with various other toxins in animal feed and agricultural commodities, i.e., aflatoxin B_1/ochratoxin (Harvey *et al.*, 1989), aflatoxin / T-2 toxin (Huff *et al.*, 1988; Harvey *et al.*, 1989), aflatoxin / kojic acid (Giroir *et al.*, 1991), aflatoxin/ cyclopiazonic acid (Smith *et al.*, 1992), aflatoxin B_1/deoxynivalenol (Huff *et al.*, 1986), and ochratoxin-A/deoxynivalenol (Kubena *et al.*, 1989). The

simultaneous presence of fumonisin in aflatoxin-contaminated corn has been reported extensively worldwide (Chamberlain *et al.*, 1993; Park *et al.*, 1996; Sydenham *et al.*, 1991a; Sydenham *et al.*, 1991b; Sydenham *et al.*, 1993). Since different mycotoxins can co-exist on the same host crop, it is not unlikely that these toxins can pose a threat synergistically as the simultaneous consumption of cereal grains, i.e., wheat, rice, and corn, is prevalent all over the world. Cereals are consumed as a staple, especially in developing countries. The health threats of these toxins and the risk of their combined effect of toxicoses cannot be ruled out. Weanling infants, and growing children with an under-developed immune system are indirectly exposed to these mycotoxins through their mother's or cow's milk and cereal-based formulas. They are of major concern compared to adult exposure.

Cancer has remained the leading cause of death in both sexes, claiming more than 6 million lives every year worldwide (Pezzuto, 1997), whereas diet-related cancers account for forty percent of total cancer incidences. Food mutagens include natural mycotoxins, alkaloids from plants, and polycyclic aromatic hydrocarbons and other byproducts in cooked meats. Mycotoxins are mutagenic, teratogenic and carcinogenic compounds and their presence in foods and feeds has been unavoidable. Aflatoxins are etiological agents in hepatocellular carcinoma (primary liver cancer).

Once the cancerous process is initiated either through environmental or dietary factors, one of the two ways to prevent cancer is through treatment with chemical agents, such as, the recently debated NSAIDS (Non-steroidal anti-

inflammatory drugs) i.e., direct-acting aspirin, to reduce both morbidity and mortality, and are considered a direct way. These effects are achieved by inhibiting cycloxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid to prostaglandins (pro-inflammatory), and can stimulate tumor cell growth and suppress immune surveillance.

Compounds consumed in our diet may also inhibit COX activity. Extensive research in the natural plant substances has identified innumerable natural anti-mutagenic components. A list of the most effective natural antioxidants includes fiber, polyphenolic compounds, vitamins (A, B, C, E), flavonoids, catechin, epigallocatechin gallate, soybean proteins, carotenoids, calcium, selenium, thiocyanates, and others (Stavric, 1994). Most of the compounds listed above are antioxidants in their function/behavior. The contribution of oxidative dioxy-ridonucleic acid (DNA) damage, mediated by reactive oxygen radicals in carcinogenesis, has recently attracted much attention (Halliwell and Gutteridge, 1989). The protection afforded by antioxidants against carcinogenesis might therefore be due to the prevention of oxidative DNA damage, and thus leads to the inhibition of carcinogenesis.

Anti-mutagenic factors whether natural or synthetic, are able to lower or abolish the genotoxic effects of mutagenic and carcinogenic substances. The best candidate to prevent cancer through its anti-mutagenic activity is a natural component. There are many natural micro-nutrient and non-nutritive substances in foods that can counteract mutagenic and carcinogenic processes. Currently, the role of plant derived dietary fibers in the protection of human mutagenesis

and carcinogenesis has been extensively studied. The major dietary fibers come from cereal grain or bran-milling fraction. Initially, the fibers were thought to dilute carcinogens through increasing the bulk of stool and decreasing the transit period in the intestine (Burkitt, 1975), but in fact, Graf and Eaton (1985) identified an additional component, phytic acid. Substantial evidence in animal studies have proved that phytate or phytic acid (inositol hexaphosphate), a component of the dietary fiber complex, reduces the risk of large intestinal cancer. It has been assumed that phytic acid, which is an intrinsic component of grains, can render protection against cancer through its antioxidant property (Shamsuddin, 1995).

Phytic acid (*inositol hexaphosphate*, $InsP_6$) is a naturally occurring compound in cereals and legumes (0.4 - 6.4%). It exists primarily as a salt with mono- and di-valent cations (Ca, Na, Mg, K) and is considered the chief storage form of phosphorous for germinating seeds. Not surprisingly, it has been considered an anti-nutritive factor due to its chelating property with important minerals (Cu > Zn > Mn > >Fe > Ca, in decreasing order), and therefore inhibits mineral absorption. Paradoxically, the ability of phytate to bind with metals especially iron (Fe^{2+}), may be responsible for phytic acid's antioxidant and anticarcinogenic activity. Phytic acid inhibits Fe-induced free radical (.OH) generation by chelating Fe^{2+} (Graf and Eaton, 1990). This is achieved by occupying all the available Fe^{2+}-coordination sites thus inhibiting .OH generation from the Fenton reaction. Subsequently, it limits the processes of

lipid peroxidation and DNA damage by inhibiting free radical formation, which is thought to be involved in the etiology of certain cancers (Shamsuddin, 1995).

Although the actual mode of action of phytic acid is not known except for its chelation and anti-oxidant properties in the meal matrices, it has been assumed that phytic acid might have the capacity to bind to the toxin(s) and/or to produce some less reactive metabolites through its binding to the hydroxylated form of aflatoxin. Phytic acid might also inhibit the biosynthesis of toxins by their respective molds through its antioxidant properties. It was also hypothesized that the presence of phytic acid along with other antimutagenic factors such as linoleic acid in corn, which is part of the initial substrate for phytic acid formation, phosphotidylinositol, would affect the formation and/or toxic/mutagenic potential of aflatoxins.

The proposed study was aimed at developing a risk management correlation between the aflatoxins and the intrinsic anti-carcinogenic components in corn. In this regard, the preliminary testing was to determine the intrinsic amount of phytic acid required to counter-react pure aflatoxin B_1. The efficacy of phytic acid and linoleic acid on toxin formation by *Aspergillus flavus* (Link ex. Fries) in inoculated corn, and the anti-aflatoxin modifying effects of phytic acid and linoleic acid in combination have also been studied.

Based on the aforementioned, the primary objective of this study was: 1) to determine the antimutagenic potential of phytic acid, linoleic acid, and phosphotidylinositol and their possible interactions against different direct acting and indirect acting carcinogens. After the determination of this objective,

the secondary objectives were: 2) to determine the role and effect of phytic acid on the production of aflatoxins from *Aspergillus flavus* Link ex. Fries in the presence and absence of metal ions during storage; 3) to determine the effect of phytic acid and linoleic acid individually and in combination on the production of aflatoxins during the storage of corn innoculated with *Aspergillus flavus* Link ex. Fries.

2. LITERATURE REVIEW

A. Historical Background

Mycotoxins are structurally a diverse group of mostly small molecular weight compounds produced as secondary metabolites of fungi. Mycotoxins are ubiquitous in a broad range of commodities and feeds and are toxic to mammals, poultry and fish. Ergotism is probably the oldest known mycotoxicosis. Human outbreaks associated with *Claviceps purpurea* contamination of rye flour were widespread in central and northern Europe in the Middle Ages, e.g., the epidemic of the year 944 A.D. in Aquitaine and Limoges in France killed 40,000 people (King, 1979). Many of the victims of the gangrenous ergotism regarded the burning sensation in their limbs as a divine punishment, and it was described as the Holy Fire, St. Anthony's Fire and *feu sacre'*. The epoch-making discovery during the 1960s of aflatoxins, a group of closely related hepato-carcinogenic metabolites, produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus,* led to the resurgence of interest in all aspects of mycotoxicology. The global nature of the mycotoxin problem is based on well-documented human mycotoxicoses such as ergotism in Europe, alimentary toxic aleukia (ATA) in Russia, acute aflatoxicoses in South and East Asia, and human primary liver cancer (PLC) in Africa and South East Asia. Ochratoxin A (OTA) is suspected of playing a role in the Balkan endemic nephropathy (BEN) of Yugoslavia and chronic interstitial nephritis (CIN) in North Africa (Steyn, 1995).

In nature, most cereal grains, oilseeds, tree nuts, and dehydrated fruits are susceptible to fungal contamination and mycotoxin formation. These toxins are produced mainly by five genera of fungi namely, *Aspergillus, Penicillium, Fusarium, Alternaria,* and *Claviceps.* Their presence in food and feed commodities is related to the climatic and other growth-related factors that influence the production of mycotoxins by these fungi. Under laboratory conditions at least 300 mycotoxins have been produced by pure cultures of fungi and chemically characterized. Fortunately, only about 20 mycotoxins are known to occur in foodstuffs at significant levels and frequency to be of food safety concern (Steyn, 1995). Their presence in food and feed commodities is related to the climatic and other growth-related factors that influence the production of mycotoxins by these fungi. These naturally-occurring toxicants are unavoidable, unpredictable and pose a unique challenge to food safety. Mycotoxigenic fungi and their control pose a serious economic impact around the world.

In structural complexity, mycotoxins vary from simple C_4-compounds, e.g. moniliformin, to complex substances such as phomopsins (Culnevor *et al.*, 1989), and the tremorgenic mycotoxins (Steyn and Vleggaar, 1985). Mycotoxins induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins, ochratoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (ochratoxins), estrogenic (zearalenone), hemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ergotoxins), hepatotoxic (aflatoxins and phomopsins), dermatoxic (trichothecenes), and neurotoxic (ergotoxins, penitrems, lolitrems and paxilline),

whereas others display antitumor, cytotoxic, and antimicrobial properties (Steyn, 1995).

Molds and mycotoxins have a considerable impact worldwide in terms of public health, agriculture and economics. The production of agricultural commodities is barely sustaining the world's increasing population and every year, at least 25% of the world's food crops are contaminated with mycotoxins (FAO, 1996). They assert discernable costs to farmers, livestock and poultry producers, grain handlers, and food and feed processors. The ubiquity of toxigenic molds, the importance of cereals (corn, rice, wheat) as a human staple and animal feed, the reports of possible co-contamination and the implication of these toxins with diverse toxicological syndromes, has provoked an increased interest in the scientific community to find the appropriate risk management and regulatory program.

B. Aflatoxins

1. Discovery

Aflatoxins are a group of closely related bis-dihydrofurano secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus* growing on agricultural commodities in the field and/or while the products are stored. The discovery of aflatoxins can be traced back to the 1960s when the outbreak of "Turkey X" disease in Southeast England, which resulted in the deaths of over 100,000 young turkeys and tens of thousands of ducklings and young pheasants (Buchi and Rae, 1969). Deaths on such a scale, together with their serious economic implications, stimulated intensive investigations and

eventually resulted in the identification and characterization of the toxic metabolites responsible for the disease and which were isolated and called "aflatoxins" (Heathcote and Hibbert, 1978). The word aflatoxin is formed from the following set up: the first letter "A" for the genus Aspergillus, the next set of three letters, "FLA" for the species flavus, and the noun "TOXIN" meaning poison (Ellis *et al.*, 1991). Chemically, aflatoxins are difuranocoumarin derivatives (Buchi and Rae, 1969). Aflatoxin B_1 (AFB_1) has a molecular weight of 312 and an empirical formula of $C_{17}H_{12}O_6$ (Pavao *et al.*, 1995). It decomposes without melting at 268-269° C (Beuchat, 1978). Presently, 18 different types of aflatoxins have been identified with aflatoxins B_1, B_2, G_1, G_1, M_1, and M_2 being the most common (Beuchat, 1987) (Pavao, 1995; Figure 2.1). Of these, B_1 and G1 occur most frequently, with B_1 having the most potent toxicity. The letters B and G refer to the most fluorescent colors (blue and green, respectively) observed under long-wave ultraviolet (UV) light and the subscripts 1 and 2, to the separation patterns of chemically similar compounds on thin layer chromatographic plates (Bullerman, 1979). The letter M for M1 and M2 refer to the milk where these two toxins were primarily identified (Bhatnagar *et al.*, 1994).

Aflatoxins are found in corn, wheat, rice, barley, cottonseed, peanuts, and other legumes. Aflatoxin B_1 (AFB_1) is the most carcinogenic of the aflatoxins to date and causes primary liver cancer (PLC) through necrosis, immune-supression, gastrointestinal tract dysfunction, and pulmonary edema in

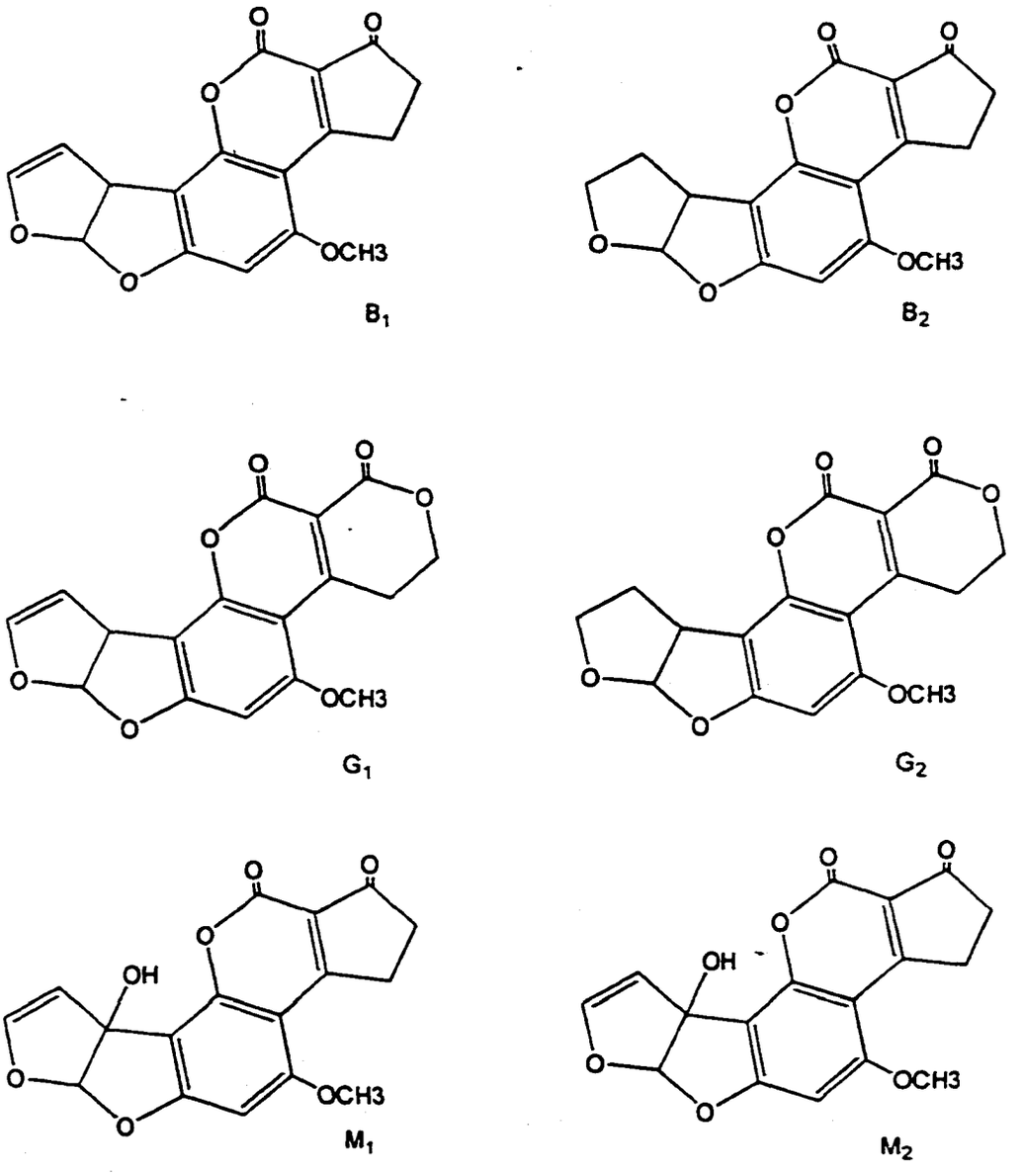

Figure 2.1 Chemical structures of naturally-occurring aflatoxins (adapted from Pavao *et al.*, 1995)

animals and humans. It is metabolized by a phase I enzyme system resulting in the production of highly reactive epoxides, which cause damage to cells by covalently binding to the proteins and DNA. The worldwide occurrence of aflatoxins coincides with high temperature and moisture conditions. Its exposure is generally considered a major etiological factor in hepatocellular carcinoma, a malignant neoplasm of hepatic cells commonly referred to as primary liver cancer. Aflatoxins (especially AFB_1) are considered as potent tumorigenic and tumor promoting agents. Most of the actions are attributed to its metabolites, which react with DNA and proteins through the cytochrome P450 enzyme system pathway. The 8,9-epoxide formation has been postulated as the ultimate tumorigenic agent. Chronic and sub-chronic (short-term) exposure to AFB_1 produces hepatic cancer.

AFB_1 was considered to be the main issue for a safer food supply until recently, but due to an advancement in analytical techniques other toxins have also been discovered. The simultaneous occurrence of aflatoxin with other mycotoxins has also been reported in corn from the United States of America (Chamberlian *et al.*, 1993), South-America (Resnik *et al.*, 1996; Julian *et al.*, 1995; Heningen and Dick, 1995), China (Wang *et al.*, 1995), Central Africa (Atawodi *et al.*, 1994; Ibeh *et al.*, 1992), and South Africa (Sydenham *et al.*, 1993). Since aflatoxins are ubiquitous in food environment and their simultaneous presence with other toxins has also been reported, the next section will address the issue related to their mycology, human exposure, metabolism and decontamination of aflatoxin-effected agricultural commodities.

2. Mycology and Occurrence

The ubiquity of *A. flavus*, as well as its ability to colonize multiple commodities has prompted extensive research for the prevention of contamination. Earlier studies assumed that invasion was a function of inadequate storage conditions (*i.e.*, drying, temperature, and moisture). However, newer studies have shown that invasion before harvest is equally or more important (Cole *et al.*, 1982; Guo *et al.*, 1995; Klich *et al.*, 1984; Lopez-Garcia, 1998; McDonald and Harkness, 1967). Corn (*Zea mays*) is an important crop in the grain and livestock economy worldwide. In corn, pre-harvest invasion is primarily dependent on insect damage of the developing cobs. The fungus can also invade by growing down the silks of the developing ears (Jones *et al.*, 1980; Lillehoj *et al.*, 1980). The ability to grow as a non-destructive pathogen in the tissues of a variety of plants over the normal range of food storage temperatures as well as the capacity to grow at low water activity give *A. flavus* the potential to grow in the vast majority of commodities, if pre-harvest, harvest and storage conditions are less than ideal.

Weather conditions also play an important role in aflatoxin formation. Since *A. flavus* is considered an opportunistic pathogen, any factor that affects the normal development of the plant would favor fungal invasion and subsequently, toxin production. Alternate cropping patterns and different irrigation techniques have been shown to reduce aflatoxin formation. Payne *et al.* (1988) reported that drought stress led to an increased number of infected kernels in silk-inoculated ears. Other environmental factors that affect toxin

production and accumulation include lack or excess of nitrogen, excessive plant populations, and poor irrigation practices (Anderson *et al.*, 1975; Fornum and Manwiller, 1985; Payne *et al.*, 1986).

The aflatoxigenic species of the *Aspergillus* group includes *A. flavus, A. parasiticus*, and *A. nomius*. *A. flavus* and *A. parasiticus* may colonize the food/feed and produce mycotoxins. *A. flavus* Link ex Fries and *A. parasiticus* Speare have been shown to produce aflatoxins on a wide variety of grains and peanuts, but have also been reported on numerous other agricultural commodities. These strains of *Aspergillus* are capable of producing aflatoxins B_1, B_2, G_1, G_2, and M_1, and other related compounds (Wilson and Payne, 1994). Currently, the taxonomy of the *A. flavus* group is uncertain and the differences between these fungi are often inconclusive, which makes it difficult to identify distinct species. According to Wicklow (1983) and Klich and Pitt (1988), *A. flavus* and *A. parasiticus* are taxonomically distinct species. However, previous reports by Hesseltine *et al.* (1963) have described several isolates with intermediate taxonomic characteristics between *A. flavus* and *A. parasiticus*. In 1986, Kurtzman and collegues complicated the matter by proposing that both *A. flavus* and *A. parasiticus* were so closely related that they should be classified as *A. flavus* var. flavus and *A. flavus* var. parasiticus. However, these classifications are only of concern to the pure taxonomist. Most of the literature refers to the toxigenic species as the *A. flavus* group without further specifications.

The most thorough investigations of seed pathogenesis have been conducted with maize kernels, in which *Aspergillus* spp. preferentially colonize the lipid-rich embryo (Brown *et al.*, 1993) and the aleurone tissues (Keller *et al.*, 1994a). Aspergilli typically gain access to the seed through cracks generated by environmental stress (heat and/or drought) or via insect damage. Several studies have described the role for specific fungal degradative enzymes (Brown *et al.*, 1993; Burow *et al.*, 1997; Cotty *et al.*, 1990), and proteinous structures (Huang *et al.*, 1997) in *Aspergillus* pathogenesis and aflatoxin biosynthesis.

Pitt and Hocking, (1997) have recently observed the growth patterns of *Aspergillus flavus* and *Aspergillus parasiticus* in Czapek yeast extract agar (CYA) showing colonies of 60-70 mm in diameter, plane, sparse to moderately dense. The colonies are characteristically greyish green to olive yellow, sometimes only yellow and become green with age. They concluded that the age of mycelium and their genotype is important in the fluorescence of aflatoxins. In various studies conducted to find the optimum growth temperature and other environmental conditions, they were able to distinguish them by their rapid growth both between 25° and 37° C. The difference between these two species is that *A. flavus* produces conidid which are variable in shape and size and have thin walls that vary from smooth to moderately rough and in contrast, *A. parasiticus* colonies are spherical with relatively thick, rough walls.

Various studies have also been conducted to optimize the environmental conditions and kernel germination (Guo *et al.*, 1995). It was observed that

growth temperatures for *A. flavus* vary from a minimum near 10-12°C, a maximum near 43-48°C, and an optimum near 33°C (Domsch *et al.*, 1980; ICMSF, 1996). The water activity needed for growth also varies in a range of 0.78 at 33°C (Ayerst, 1969) to 0.84 at 30°C and 0.80 at 37°C (Pitt and Hocking, 1977). These molds can grow in a very wide range of pH. It has been observed that growth can occur from pH 2.1 to 11.2 at 25°, 30° and 37°C. Optimal growth was recorded from pH 3.4 to 10 and a peak near 7.5 (Wheeler *et al.*, 1991; Olutiola, 1976).

A. flavus has also been adapted for use on a broad assortment of organic resources as a source of nutrients. It is considered a saprobe, as well as an opportunistic pathogen of plants, insects, domestic animals and humans. In the field, *A. flavus* population increases during hot and dry conditions. Crop debris, dormant tissues, and/or damaged and weakened crops are their primary substrates for growth (Asworth *et al.*, 1969; Stephenson and Russell, 1974).

3. Toxin Production

Aflatoxins B and G are produced by all toxigenic strains of *A. parasiticus*. On the other hand, most, but not all *A. flavus* isolates produce only the B aflatoxins (Pitt, 1989). In general, commodities containing the B-type aflatoxins may be contaminated with *A. flavus* only. However, there is no evidence that suggests that *A. parasiticus* is the only source of the G aflatoxins (Wilson and Payne, 1994)

Some strains of *A. flavus* produce cyclopiazonic acid (CPA) (Trucksess *et al.*, 1987). This toxin has been reported to be toxic in rats, dogs, pigs, and chickens (Lomax *et al.*, 1984; Nuehring *et al.*, 1985). CPA has metal chelating properties, and has been reported to bind physiolgically important cations such as calcium, magnesium and iron (Gallagher *et al.*, 1978). CPA occurs naturally in corn, peasnuts, cheese and millet and it can be found with aflatoxins (Gallagher *et al.*, 1978; Trucksess *et al.*, 1987). Due to this co-contamination, the toxicity of commodities contaminated with *A. flavus* can be increased due to the presence of other metabolites than expected from the levels of aflatoxins. The same phenomenon was observed during the biosynthesis of aflatoxins by Wong *et al.*, 1977. They reported the presence, persistence and mutagenicity of versicolorin A and sterigmatocystin, the fungal intermediate metabolites, during aflatoxin biosynthesis. The role of these intermediate metabolites and interaction of CPA and aflatoxin should be explored to further understand the toxicological implications of consumption of foods/feed contaminated with *A. flavus*.

4. Chemical Characteristics

Aflatoxins are a group of bisfuran coumarin compounds produced by mold secondary metabolism and have no obvious physiological role in the primary growth and function of the mold (Bhatnagar, 1991). However, byproducts of primary metabolism are generally precursors of secondary metabolites. Thus, primary and secondary metabolites are intricately related

(Drew and Demain, 1977). Aflatoxin biosynthesis is related to mold lipid biosynthesis (Townsend *et al.*, 1984).

Aflatoxin B_1 is one of the most potent naturally occurring carcinogens. Chemically, it is a carbonyl group in a five-membered ring and cross-conjugated with an alpha, beta-unsaturated function. Its formula is C_{17} H_{12} O_6 with a molecular weight of 312 (Pavao *et al.*, 1995). Aflatoxins are soluble in a number of non-polar compounds and insoluble in water. Each of the known aflatoxins has a different structure with variable activity. The names of the most common aflatoxins, B and G for blue and green respectively refer to their fluorescent colors under ultraviolet light. In the case of aflatoxin M, an important aflatoxin metabolite, the letter refers to its presence in milk (Bhatnagar *et al.*, 1994).

Its basic structure is derived from acetate units from the polyketide pathway. Bhatnagar *et al.* (1991) have suggested that AFB_1 biosynthesis is based on a C_{20} polyketide (decaketide) precursor. Polyketides are a large group of structurally diverse compounds that arise from the head-tail condensation of acetate units (Applebaum and Marth, 1981). These molecules then undergo several transformation reactions where the chain is folded, condensed, oxidized, reduced, cleaved and rearranged to produce a wide variety of molecules, including the aflatoxins (Bhatnagar, 1991). Several studies have reported that the degradation of aflatoxins takes place in old or aging mycelium (Hamid and Smith, 1987a; Huynh and Lloyd, 1984). The involvement of fungal cytochrome P-450 (CP-450) monooxygenase enzyme systems in the degradation of aflatoxin

B_1 and G_1 by intact mycelium and cell-free extracts of *A. flavus* has also been observed (Hamid and Smith, 1987b). The role of CP-450s on AFB_1 formation, degradation, and its epoxidation pathways has been extensively reported in different animal models and human liver (Guengerich *et al.*, 1998; Pelkonen *et al.*, 1997; Roy and Kulkarni, 1997).

5. Metabolism

a. Absorption and distribution

Human exposure to aflatoxins occurs mainly via ingestion of contaminated foodstuffs and through prolonged occupational exposure. Therefore, most of the pharmacokinetic considerations are associated with the chronic administration of relatively low doses of AFB_1 through the oral route (Hsieh and Wong, 1994). AFB_1 is a relatively low molecular weight (312) lipophilic compound. This suggests that its absorption after ingestion is efficient. Wogan *et al.* (1967) reported that absorption after oral administration of 0.07 mg/kg [^{14}C] labeled AFB_1 in male Fisher rats was complete as compared to intraperitoneal administration. They observed that up to 20% and 60 % of the original dose was eliminated via urinary and fecal routes, respectively. Slow passage through the intestinal tract was noted since little radioactivity was excreted in the first 8 hours. After 24 hours, the residual radioactivity in the carcass (7.6% of the original doses) was found in the liver, indicating that it is the primary site of accumulation of AFB_1, its metabolites and/or bound material.

With regard to AFB_1 absorption, it has been reported that duodenum is most efficient site for AFB_1 absorption where it is rapidly absorbed from the

small intestines into the mesenteric venous blood (Kumagai, 1989). This study suggested that species differences in AFB_1 uptake could be due to differences in the composition of the intestinal epithelium. The author also reported that the degree of aflatoxin uptake is proportional to AFB_1 concentration indicating that it is absorbed by passive diffusion. AFB_1, a less lipophilic analog, was absorbed at a lower rate, indicating the importance of lipophilicity in aflatoxin absorption.

Excretion of AFB_1 occurs primarily through the biliary pathway, followed by the urinary pathway. From the intestine, AFB_1 apparently enters the liver through the hepatic portal blood supply (Wilson *et al.*, 1985). The liver readily concentrates the toxin. Given the high efficiency of the liver to extract free AFB_1 from the blood, the binding of AFB_1 to serum albumin at the site of intestinal absorption has not been considered a major mechanism of detoxification. The kidneys have been shown to concentrate aflatoxin to a lesser extent (Hsieh and Wong, 1994).

b. Phase I metabolism

AFB_1 is considered the most potent of the aflatoxins and related benzofurans in most genotoxicity assays; it is also the most hepato-carcinogenic (Busby and Wogan, 1984). However, all evidence available to date proves that AFB_1 by itself is not particularly genotoxic, and that the metabolically produced *exo* isomer of epoxide is the only genotoxic product. It is evident that the genotoxic response has been due to either base-pair mutations, frameshift mutations or induction of bacterial SOS response (Baertschi *et al.*, 1989; Busby and Wogan, 1984; Garner *et al.*, 1972). The stereochemistry of AFB_1

epoxidation is of considerable significance in determining the course of its toxicity (Johnson *et al.*, 1996).

Cytochrome P-450 (P450) enzymes are involved in the oxidation of numerous steroids, eicosanoids, alkaloids, and other endogenous substrates. These enzymes also have a major involvement in the oxidation of potential toxicants and carcinogens such as those encountered among environmental pollutants, solvents, pesticides and many natural products like aflatoxins (Guengerich, *et al.*, 1996). P450 enzymes produce two different sterometric forms of the 8,9 epoxide, the *exo*-8,9 epoxide in which the ring is positioned "below" the plane and the *endo*-8,9 epoxide in which the ring is positioned "above" the plane. Of these, the *exo* isomer is at least 1000 times more genotoxic than its *endo* counterpart (Guengerich, *et al.*, 1996). The bioactive form of aflatoxin is considered a strong electrophile that can form covalent adducts with macromolecules such as proteins, RNA and the N-7 position of guanidine residues in DNA (Figure 2.2) (Foster *et al.*, 1983; Miller, 1991).

Cytochrome P450 isozymes produce a number of products that can be considered activated metabolites (*i.e.*, 8,9 epoxide) or detoxification products, such as aflatoxins M_1, P_1 and Q_1 (Figure 2.3) (Langouet *et al.*, 1996). Although peroxidases and lipoxygenases have been reported to oxidize AFB_1 (Battista and Marnett, 1985), the P450s, CYP3A1, CYP1A2, CYPIA1 and CYP3A5 have been shown to transform AFB_1 as well. Cytochromes 1A2 and 3A4 have been considered the most active. However, there is controversial information as to which is the dominant enzyme. Ueng *et al.* (1995) have reported that CYP3A4

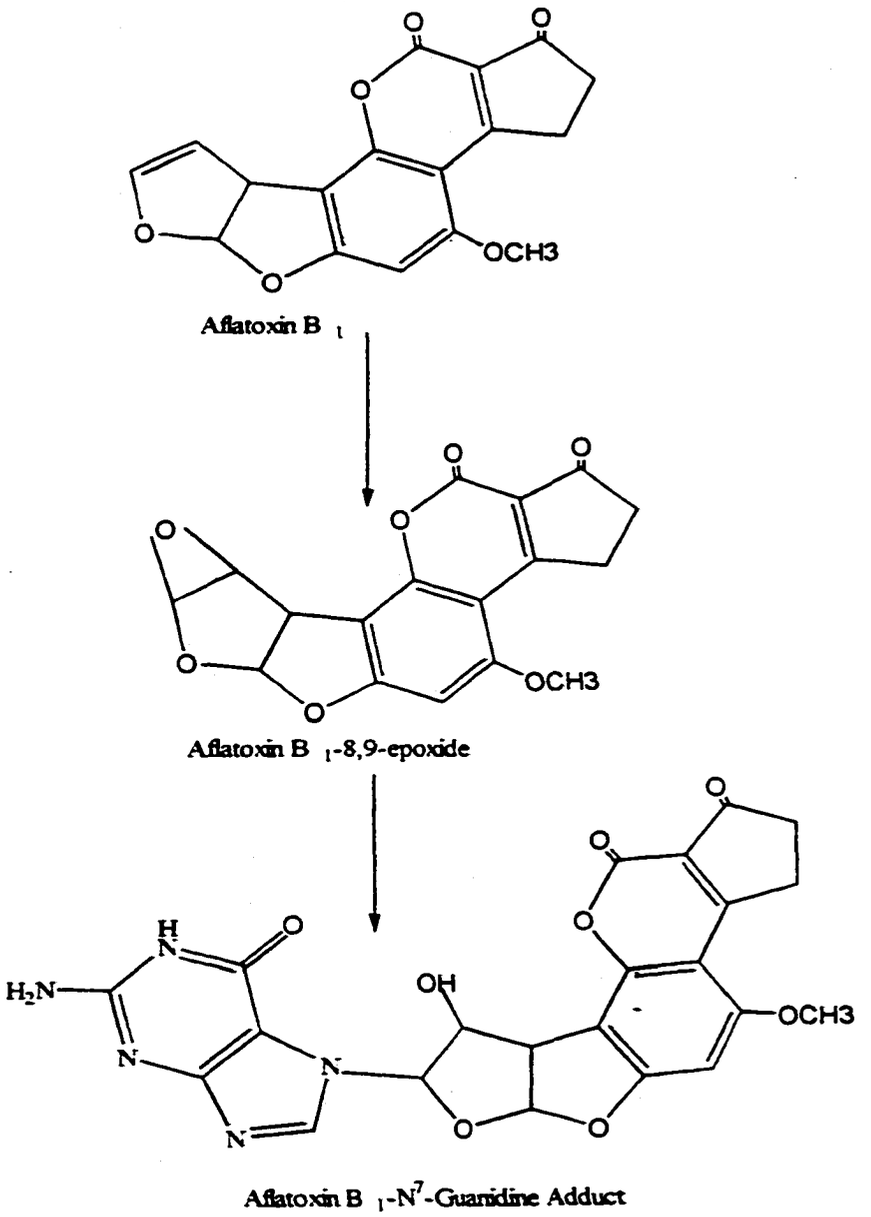

Figure 2.2 Formation of aflatoxin B_1-N7 guanidine adduct (adapted from Pavao *et al.*, 1995)

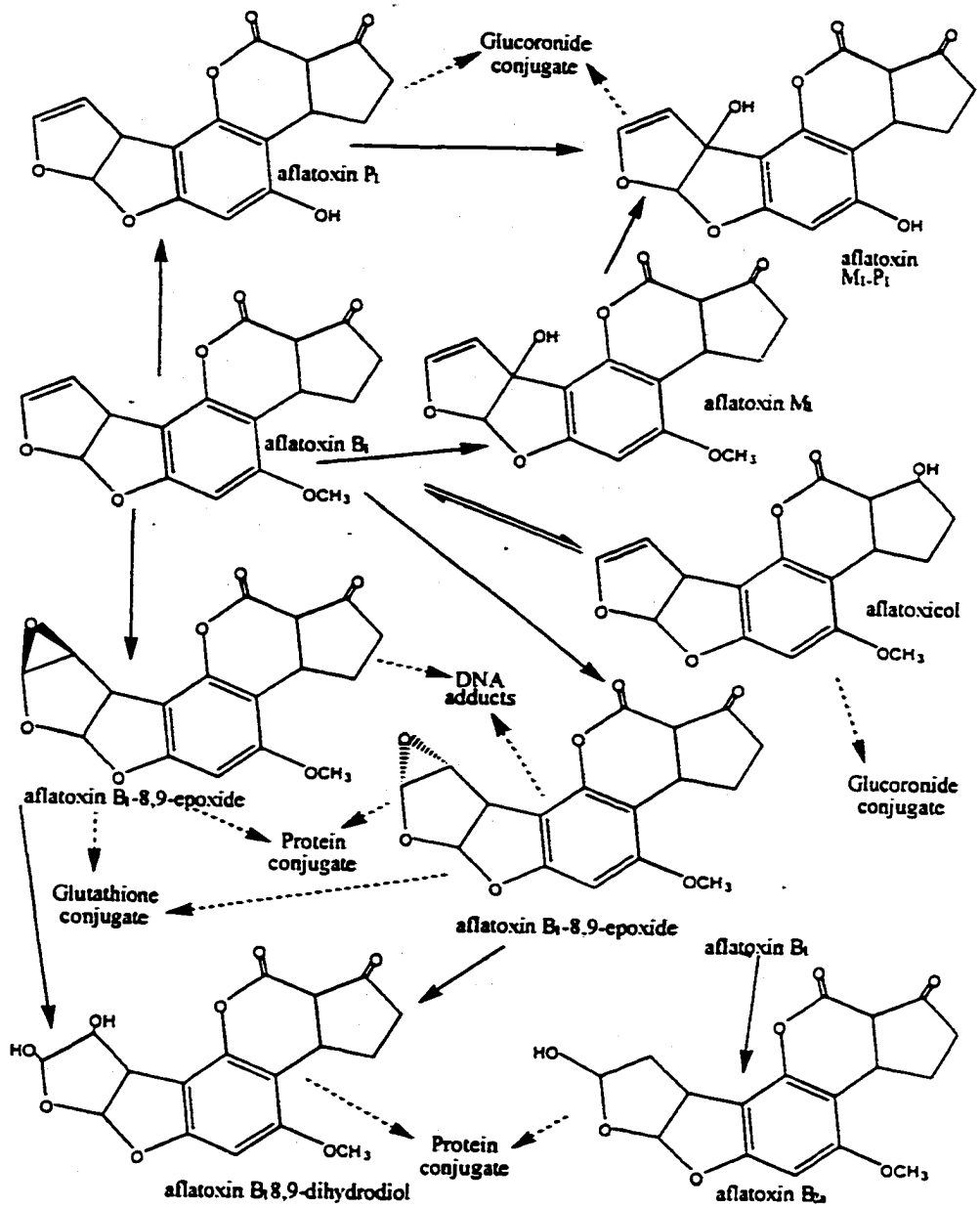

Figure 2.3 Biotransformation pathways of AFB_1 (adapted from Lopez-Garcia, 1998)

appears to be the enzyme involved to the greatest extent in AFB_1 activation. Further supporting evidence to this hypothesis has also been provided by Forrester *et al.* (1990) and Aoyama *et al.* (1990). However, Gallagher *et al.* (1996) reported that CYP1A2 contributes to over 95 % of AFB_1 activation in human liver microsomes at 0.1mM AFB_1. The discrepancies between these studies may be a result of different sources of P450's used among the different experiments. The reconstitution system of Ueng *et al.* (1995) was rather complex and cannot be considered similar to the *in vitro* system employed by Gallagher *et al.* (1996). Also, the source of the human CYP1A2 cDNAs used in the experiments of Ueng *et al.* (1995) contained a modified N-terminus (Sandhu *et al.*, 1994) and therefore may have exhibited somewhat different catalytic activities from the CYP1A2 cDNA - expressed microsomes used in the Gallagher *et al.* (1996) study. This study also reported kinetic models that support CYP1A2's dominant role in AFB_1 metabolism *in vivo*.

c. Phase II metabolism

Phase II metabolism leads to the conjugation of phase 1 metabolites to make them less toxic and more available for excretion. Species differences in the susceptibility to the toxicity and carcinogenicity of AFB_1 are determined largely by the organism's ability to biotransform the toxin. The major phase II metabolites are the glutathione, glucoronide and sulfate conjugates (Hsieh and Wong, 1994). The glutathione (GSH) conjugate of the AFB_1 8, 9 epoxide has been identified as the major phase II reaction. Thus, any particular species sensitivity has been related to its ability to conjugate the epoxide with GSH.

There is some evidence that indicates that the various glutathione-S-transferases have differential activity toward the *endo* and the *exo* forms of the 8,9 epoxide.

Glucoronide conjugation also plays a role in biotransformation and excretion of AFB_1 and /or its metabolites. Loveland *et al.* (1984) reported that glucoronides of aflatoxicol and aflatoxicol-M_1 are the principal biliary metabolites of AFB_1 in trout. Phase I metabolites such as aflatoxins P_1, Q_1, and M_1 go through glucoronide conjugation. It has been shown that the rate of conjugaton of these three metabolites differ because of different types of hydroxyl groups (Metcalfe and Neal, 1983). The phenolic hydroxyl group present in AFP_1 is a much better site for glucoronide conjugation than the hydroxyl groups present in AFM_1 and AFQ_1.

d. Competitive pathways: activation and detoxification

The fate of AFB_1 in an organism depends on a delicate equilibrium between several pathways. The quantity of aflatoxin available to exert toxic, mutagenic and carcinogenic effects will depend on the amount converted to the various metabolites and their relative biological activity. Thus, the hydroxylated compounds, i.e., AFM_1, AFP_1 and AFQ_1, are considered detoxification products due to their lower ability to react with DNA and proteins. The 8,9 epoxide is not always a toxic product since detoxification of this reactive molecule may occur through phase II conjugation with gluatathione. Hydrolysis of the electrophilic epoxide to form a dihydrodiol also represents a decrease in toxic potential. The dihydrodiol can exist in a resonance form as a phenolate ion that is capable of forming Schiff base adducts with protein amino groups, particularly lysine (Lin

et al., 1978). Even if the binding to protein can represent a toxicological risk, the relative toxic, mutagenic and carcinogenic effects of protein conjugation is much lower than that of the active epoxide.

It is difficult to discuss AFB_1 activation and detoxification pathways without considering the kinetics involved in each reaction. Unfortunately, most *in vitro* studies use AFB_1 concentrations that surpass the actual dietary human exposure. It has been noted that humans and animals get chronically exposed to low doses rather than to one acute single dose. Thus, it is difficult to extrapolate from *in vitro* studies. Another factor to consider is that the activity of the different metabolizing enzymes varies from species to species. Ramsdell and Eaton (1990) demonstrated substantial variations in the patterns of liver microsomal AFB_1 oxidation over a range of concentrations for four species: rat, mouse, monkey and human. Affinity to detoxification reactions also plays an important role in AFB_1's ultimate toxic effect. As with microsomal activation, the affinity of the enzyme glutathione S-transferase to the AFB_1-8,9-epoxide varies across species.

The information available to date suggests that the ultimate toxic response to aflatoxin is not a univariate process. It is a multivariate equilibrium that depends on external factors, such as toxin concentration, presence of other xenobiotics, environmental stress and nutritional status among others and on internal factors that include but are not limited to genetic predisposition, type and activity of microsomal enzymes, and availability of efficient detoxification mechanisms.

6. Toxicity

The liver is considered the primary target organ for aflatoxin toxicity. Since its characterization in the early 1960's, acute structural and functional damage to the liver has been reproduced in wide variety of species. Acute aflatoxicosis has been characterized by vomiting, abdominal pain, pulmonary edema, fatty infiltration and necrosis of the liver (Shank, 1981). Although there is ample evidence for substantial human exposure in certain populations, information on clinical aflatoxicosis in humans is still limited (Busby and Wogan, 1984). A review of the effects of acute exposure to aflatoxins reveals that a wide variety of vertebrates, invertebrates, plants, bacteria, and fungi are sensitive to these toxins, but the range of sensitivity is wide. The basis for the species and strain variation in the acute toxicity of aflatoxin is not fully understood. As discussed in the previous section, two important factors in aflatoxicosis are: (1) the proportion of AFB_1 that is metabolized to the 8, 9 epoxide relative to other metabolites that are considerably less toxic and; (2) the relative activity of phase II metabolism, which forms non-toxic conjugates and inhibits cytotoxicity (Cullen and Newberne, 1994).

The aflatoxins have been studied extensively *in vitro* (IARC, 1987). It has been reported that all the members of the family (AFB_1, AFB_2, AFG_1, and AFG_2) produce genotoxic injury. Among them AFB_1 is considered the most potent genotoxic agent *in vitro* and *in vivo*, whereas AFG_2 the least potent. All of them are naturally-occurring aflatoxins, as well as, the metabolite AFM_1, have been reported to produce some injury to DNA. AFB_1 produces chromosomal

aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, and chromosomal strand breaks, and form adducts in rodent and human cells (IARC, 1987). AFG_1 produces chromosomal aberrations in Chinese hamster bone marrow cells *in vivo*, as well as chromosomal aberrations and sister chromatid exchange in Chinese hamster cells *in vitro*. AFB_2 binds covalently to DNA of rat hepatocytes, produces sister chromatid exchange in Chinese hamster ovary cells, and stimulates unscheduled DNA synthesis in human fibroblasts *in vitro*. Unscheduled DNA synthesis in rat and in Chinese hamster hepatocytes results following exposure to AFG_2 and AFM_1 *in vitro*.

The aflatoxins have been examined for potency in a range of primary cultures, as well as, in established cell lines. Liver cell cultures from chick embryos demonstrate cytotoxicity of AFB_1 to both mesenchymal and parenchymal cells (Terao, 1967). Zuckerman *et al.* (1967) reported that 1 mg/ml exhibited cytotoxic effects to 50% of human embryo liver cells following 24 hours exposure and electron microscopy revealed nucleolar capping of the chromatin, rounding of the cell, and granulation of the endoplasmic reticulum.

Experimental studies of aflatoxin-induced carcinogenesis support the paradigm of cancer as a multistage process. Aflatoxin is considered a potent tumorigenic agent, but it has also been shown to have promoting ability (Newberne and Butler, 1969; Busby and Wogan, 1984). Virtually all the toxic effects of AFB_1 are now recognized to be attributable to the action of its electrophilic metabolites on macromolecules (DNA and proteins). Aflatoxin carcinogenesis can be promoted by a variety of factors, including an enhanced

proliferative rate and alterations in dietary intake (Rogers, 1994). Since Wogan (1966) reported that continuous lifetime exposure to aflatoxin was unnecessary for the development of tumors, shorter-term exposure to aflatoxin has been used to assess the risks associated with this toxin. The early studies of aflatoxin-induced liver carcinogenesis in rats indicated the presence and sequential development of precursor lesions (Lancaster *et al.*, 1961). Chronic and subchronic exposure to AFB_1 is an efficient method to produce hepatic cancer. For example, 2 cycles of 5 days each duration with 25 mg AFB_1 per day to young (approximately 100g) Fisher 344 rats yielded putative pre-neoplastic foci at 2-3 months postdosing (Appleton and Campbell, 1983; Kensler *et al.*, 1992; Roebuck *et al.*, 1991), while the hepatic cancers arise by approximately 1 year postdosing (Roebuck *et al.*, 1991).

C. Detoxification/Decontamination Procedures for Aflatoxin

The ever-increasing number of reports on the presence of mycotoxins in foods and feeds dictates the exigency for practical and economical detoxification procedures. Such methods should not only reduce the concentrations of toxins to safe levels but they should also not produce toxic degradation products nor reduce the nutritional value of the treated commodity. Obviously, the best approach to control human and animal exposure to aflatoxin contamination is by simple prevention (Park, 1993); however, this is not always possible, especially before harvest. A number of approaches have been taken to detoxify mycotoxins; however, only a few have apparent practical applications. For

purposes of discussion, the means of degrading mycotoxins will be grouped into three categories: a) physical; b) chemical; and c) biological.

1. Physical Degradation

In addition to the physical methods based on manual or mechanical separation, the physical degradation/decontamination procedures are carried out by using absorption, irradiation, and heat treatments.

Aflatoxin is quite stable to heat. Peers and Linsell (1975) reported the degradation of aflatoxin AFB_1 in peanut and corn oil at an optimum temperature of 250° C. However, the moisture content of the heated product is a critical factor as the presence of moisture contributes to the rate at which heat can degrade aflatoxin. Mann *et al.* (1967) studied the effect that heat and moisture had on aflatoxins in oil seed meals. They reported that increasing the moisture content of the meal resulted in the elevated rates of aflatoxin degradation. They observed that 85% of the toxin was degraded in the meal containing 30% of moisture as compared to 30% degradation in the meal containing less than 7% moisture. In general, temperatures higher than 100° C are required for the partial degradation of aflatoxin, but these processes are discouraged due to the possibility of generating toxic pyrolysates in food and the efffect of heat on degrading other nutrients and the organoleptic properties of food itself.

Although aflatoxins are sensitive to ultraviolet light, practical usage of that treatment for destroying aflatoxins is questionable (Prasanna *et al.*, 1975; Feuell, 1966). They observed that the level of exposure to x-rays, gamma rays

and electron irradiation would also destroy the irradiated commodity. The ineffectiveness of this treatment may be explained by the fact that complex organic substances, such as aflatoxin are seldom attacked by gamma rays directly. Instead, an indirect effect usually occurs in which the radiolysis of water or other simple compounds yields free radicals, which then react with the organic molecules. The presence of moisture is likely to be a major factor in determining the effectiveness of this process.

The role of absorption in the elimination of aflatoxin from the contaminated media has been reported as an effective means of decontaminating solutions. The use of bentonite clay in the removal of aflatoxin from the liquid mediums such as milk has been reported by Masimango *et al.*, 1978. Aflatoxin M_1, a hydroxylated form of aflatoxin B_1, appears in small amounts in milk, produced by lactating cows consuming feed contaminated with aflatoxins. Applebaum and Marth (1980) have reported that the amount of absorbed aflatoxin M_1 increased as more bentonite was added to the milk. However, additional tests are needed to develop a practical means for using bentonite to remove aflatoxin from milk. In addition, the use of charcoal and charcoal with filtration has been studied to be a useful technique for removing patulin from apple cider.

2. Chemical Degradation

A variety of chemicals capable of degrading aflatoxins have been studied extensively and is a more practical approach to detoxify aflatoxins.

Aflatoxins are degraded by aqueous solutions of strong acids and bases. Strong acids catalyze the addition of water to aflatoxin B_1 and G_1 yielding a hydroxy analog of aflatoxin B_2 and G_2, commonly called B_{2a} and G_{2a}. Unfortunately, relatively drastic conditions are necessary to convert large amounts of B_1 and G_1 to B_{2a} and G_{2a} (Pons *et al.*, 1978; Doyle and Marth, 1978). Hence, it is not likely that the use of acid solution is a viable alternative for detoxifying agricultural commodities.

A variety of organic and inorganic bases have also been evaluated for their effects on aflatoxins. Chemicals such as chlorinating compounds (chlorine dioxide, sodium hypochlorite) and oxidizing agents (hydrogen peroxide, ozone, and sodium bisulfite) have been evaluated for their detoxifying properties (Samarajeewa *et al.*, 1990; Park *et al.*, 1981). Several methods, i.e., the use of chemicals $Ca(OH)_2$ (Park *et al.*, 1981), ammoniation of contaminated corn (Park *et al.*, 1988), nixtamalization in corn tortillas (Price and Jorgensen, 1985), and modified nixtamalization ($Ca(OH)_2$+ H_2O_2 and $NaHCO_3$) (Lopez-Garcia, 1995), have been developed. Samarajeewa *et al.* (1990) reported the relative efficiencies of various alkalis to destroy AFB_1 in liquid media at 110° C are: ammonium carbonate < sodium bicarbonate < ammonium hydroxide < potassium bicarbonate < sodium carbonate < potassium carbonate < sodium hydroxide < potassium hydroxide.

Bisulfite is an acceptable and commonly used food additive that is employed as a preservative in beverages, fruits, and vegetables. It may be added

to foods for a variety of reasons as it inhibits both enzymatic and non-enzymatic browning, acts as an antioxidant and as a reducing agent, and retards the growth of microorganisms (Roberts and McWeeny, 1972; Doyle and Marth, 1978a; Doyle and Marth, 1978b). In addition to these functions, bisulfite can degrade aflatoxins $B_1$and G1. Using an aqueous solution with a pH of 5.5, 50% of the aflatoxin B_1 and G1 was degraded at 25°C. Increasing the temperature from 25° to 55°C resulted in a marked increase in the rate of aflatoxin degradation (Doyle and Marth, 1978a; Doyle and Marth, 1978b). Experiments have also been conducted to evaluate sulfite for the inactivation of aflatoxin M1 in milk; however, more studies are needed to demonstrate the safer use of sulfites in detoxification.

Ammoniation appears to have generated the greatest interest as it is an effective and economically feasible means for reducing the aflatoxin from food and feed stuff (Park *et al.*, 1988). Ammonia can be delivered or applied to the treating commodity in the form of gas or aqueous solution and both have been found to equally decontaminate about 99% of the aflatoxins without affecting the proteinous contents in peanut and oilseeds (Park *et al.*, 1988). The detoxification products of ammoniation products and their distribution have also been studied (Haworth *et al.*, 1989; Park *et al.*, 1988; Weng *et al.*, 1997).

3. Biological Degradation

The use of microorganisms in food preparation, processing and preservation has been in vogue since time immemorial. Mycotoxins can also be

degraded by a variety of microorganisms. A variety of bacteria, molds and yeast can degrade or remove aflatoxin from a solution. During the screening of over one thousand microorganisms for their ability to either destroy or transform aflatoxin B_1, Ciegler et al. (1966) found one bacterium *Flavobacterium aurantiacum* that could irreversibly remove aflatoxin B_1 from an aqueous solution.

A number of microorganisms are capable of transforming AFB_1 to other characterized and yet to be characerized compounds. The list of these microbes include: *Corynebacterium rubrum, Aspergillus niger, Trichoderma viride, Mucor ambiguus, Dactylium denroides, Mucor griseo-cyanus, Absidia repens, Helminthosporium sativum, Mucor alternans, Rhizopus arrhizus, Rhizopus oryzae, Rhizopus stolonifer,* and the protozoan *Tetrahymena pyriformis* (Cole *et al.*, 1972; Detroy and Hesseltine, 1968; Mann and Rehm, 1976; Robertson *et al.*, 1970; Teunisson and Robertson, 1967). Although the conversion products in most of the studies is aflatoxicol and which is 18 times less toxic than aflatoxin B_1, the conversion of aflatoxins to aflatoxicol is slow and incomplete.

Molds that are capable of producing aflatoxins may also degrade them. A number of investigations have been carried out to optimize the production of aflatoxins from *Aspergillus flavus* and *Aspergillus parasiticus* in various media (Ciegler *et al.*, 1966; Davis et al., 1966; Diener and Davis, 1966; Doyle and Marth, 1978a; Doyle and Marth, 1978b; Doyle and Marth, 1978c; Doyle and Marth, 1979; Egel *et al.*, 1994; Hamid and Smith, 1987a & b; Hayes *et al.*, 1966; Pons *et al.*, 1972; Shih *et al.*, 1974; Yen and Lee, 1996). In an attempt to

elucidate the means by which toxigenic *Aspergillus* degrade the same aflatoxins they produce, Doyle and Marth studied many of the conditions in a series of experiments that govern degradation of aflatoxin by these molds (Doyle and Marth, 1978a; Doyle and Marth, 1978b; Doyle and Marth, 1978c; Doyle and Marth, 1978d; Doyle and Marth, 1979). They observed that the ability of Aspergilli to degrade aflatoxin was dependent on: (a) the age of mycelia:– 8 to 10-day-old mycelia were generally most effective in degrading aflatoxin B_1 (Doyle and Marth, 1978b); (b) disruption of mycelia:– blended or fragmented mycelia actively degraded aflatoxin while intact mycelia did not (Doyle and Marth, 1978a; (c) the substrate used to produce the mycelia:– substrates that support substantial growth of mycelia yield mycelia having the greatest ability to degrade aflatoxin (Doyle and Marth, 1978b); (d) the strain of *A. parasiticus* or *A. flavus*:– strains that produced larger amounts of aflatoxin generally degraded more aflatoxin; (e) the amount of mycelia in the reaction mixture:– the rate of degradation increased as the amount of mycelia was increased (Doyle and Smith, 1978d); (f) the amount of aflatoxin in the reaction mixture:– the rate of degradation of aflatoxin increased after a certain limit; (g) temperature:– maximum activity occurred at 28° C; and (h) pH:- maximum activity occurred at pH 5-6.5 (Doyle and Marth, 1978).

Other observations made by these studies included: (a) steaming the mold mycelium for 6 min resulted in a loss of ability to degrade aflatoxin; (b) broth from cultures of *A. parasiticus* was not able to degrade aflatoxin; and (c)

the supernatant fluid from homogenates of mycelia degraded aflatoxin. These results suggested that a heat-labile, intracellular factor was responsible for degrading the toxin.

Although the results from studies on conditions affecting degradation of aflatoxins by mold mycelia suggested that an enzyme might be involved, the rates at which aflatoxins were degraded did not support the possibility that the toxin was being degraded directly by enzymatic activity. Hence, it was speculated that one or more enzymes produce end products or by-products that react with aflatoxins. Peroxidase may be such an enzyme as it catalyzes decomposition of hydroperoxides resulting in the generation of free radicals (Gardner, 1970; Richardson, 1976; Liu *et al.*, 1998). These free radicals may react with aflatoxin. Furthermore, some peroxidases, such as myeloperoxidase, produce hypochlorite and singlet oxygen when in the presence of hydrogen peroxide and chloride ion (Allen, 1975). Several investigations have demonstrated that aflatoxins are effectively destroyed by hypochlorite. The mechanism by which singlet oxygen may react with aflatoxin has been described earlier.

In another study to determine the relationship between peroxidase activity of *A. parasiticus* and its ability to degrade aflatoxin, Doyle and Marth, (1978d) showed the amounts of peroxidase activity and amounts of aflatoxin B_1 and G_1 degraded by the supernatant fluid from homogenates of mycelia of *A. parasiticus*. Substantial concentrations of fungal peroxidase were present when aflatoxin was degraded.

Comparisons were then made between the ability to degrade aflatoxin and peroxidase activity in factions of crude protein obtained from supernatant fluids of mycelia that were treated with different concentrations of ammonium sulfate (Applebaum and Marth, 1980). There was no direct correlation between the amount of peroxidase activity in a particular fraction and the amount of aflatoxin degraded; however, no substantial amount of peroxidase activity was present in those fractions that also degraded substantial amounts of aflatoxin. Further support for the involvement of peroxidase in the degradation of aflatoxin is that lactoperoxidase can bring about the reactions needed to degrade aflatoxin (Applebaum and Marth, 1980; Doyle and Marth, 1978g). Degradation products of aflatoxin B_1 resulting from lactoperoxidase activity included aflatoxin B_{2a} plus water-soluble substances that were not identified. Similar products of aflatoxin B_1 degradation were produced by mycelia of *A. parasiticus* (Doyle and Marth, 1978g). However, further research is needed to confirm this relationship, as these data do not conclusively identify peroxidase activity as being responsible for the degradation of aflatoxin.

A number of approaches have been taken to remove mycotoxins from foods and feeds; however, no single treatment has proven completely successful in degrading or removing toxin(s) and retaining the nutritional and functional qualities and the treated commodity. Currently ammoniation appears to be the most promising treatment for destroying aflatoxins in feedstuffs; however, ammonia would be of limited or no value for eliminating aflatoxins from foods because of the byproducts of ammoniation process. The possible use of bisulfite

to degrade aflatoxin in food needs to be investigated further as does the use of adsorbants which may physically remove mycotoxins in general without destroying the nutritional and functional properties of the treated food. Although mycotoxins can be degraded by microorganisms, the only apparent practical application where biological degradation may be an effective means to reduce the amounts of mycotoxins in foods is the removal of patulin from apple juice by actively fermenting yeasts.

D. Other Risk Management Procedures for Aflatoxin

Since the discovery of mycotoxins, especially AFB_1, continuous concerted efforts have been made to reduce the risks related to contaminated food and feed crops. There have been some other means discussed in literature, i.e., to convert AFB_1 to a less toxic metabolite (AFM_1) in milk through feed for lactating dairy cattles (Price *et al.*, 1985). The development of host-resistant varieties of cereal crops has also been reported (Brown *et al.*, 1995; Campbell and White, 1995a, 1995b; Cotty,1994; Cotty,1990; Cuero and Osuji, 1995).

Although the risk of exposure to mycotoxins, and especially the aflatoxins, has decreased in most of the developed countries by various legislation and the stipulation for permissible levels of the toxins in human foods and animal feed, the effective control measures for the contamination of food crops by fungi and their metabolites are still inadequate. There are some legal limits for aflatoxins in both human and animal feeds in the developed countries, such legislation is uncommon in developing countries, where mycotoxin contamination is a serious problem partly because such efforts may seriously

compromise the supply of a staple. Therefore, there is the likelihood of persistent exposure to mycotoxins resulting in higher incidences of associated diseases as has been reported extensively in the literature. The safest and more feasible way to reduce the risk of cancer would be through the introduction of dietary counter-mutagens/anti-mutagens in human food and animal feeds.

E. Cancer and its Prevention

Cancer is the leading cause of death in mankind worldwide, claiming more than 6 million lives annually. Dietary habits are regarded as the possible causative factor in the development of a considerable proportion (40%) of human cancers, although these observations have not been adequately explained (Pezzuto, 1997), besides different kinds of pollutions and microbial contamination.

Carcinogenesis is a multistep process consisting of three stages: initiation, promotion, and progression. Initiation takes a short period of time and is characterized by an irreversible alteration of the cellular DNA and it allows the transformation of the cell to a nonmalignant state. Promotion takes a longer period of time and may be reversible, and it permits the nonmalignant cell to become malignant. The progression stage involves the growth of malignant cells to tumor (Thompson, 1994). Cancer is generally regarded as a disease caused by the accumulated alteration of genes in somatic cells. (Figure 2.4).

Mycotoxins, structurally, are a diverse group of mostly smaller molecular weight compounds, and are produced by the secondary metabolism of fungi and are unavoidable (Jalinek *et al.*, 1989). The Food and Argiculture

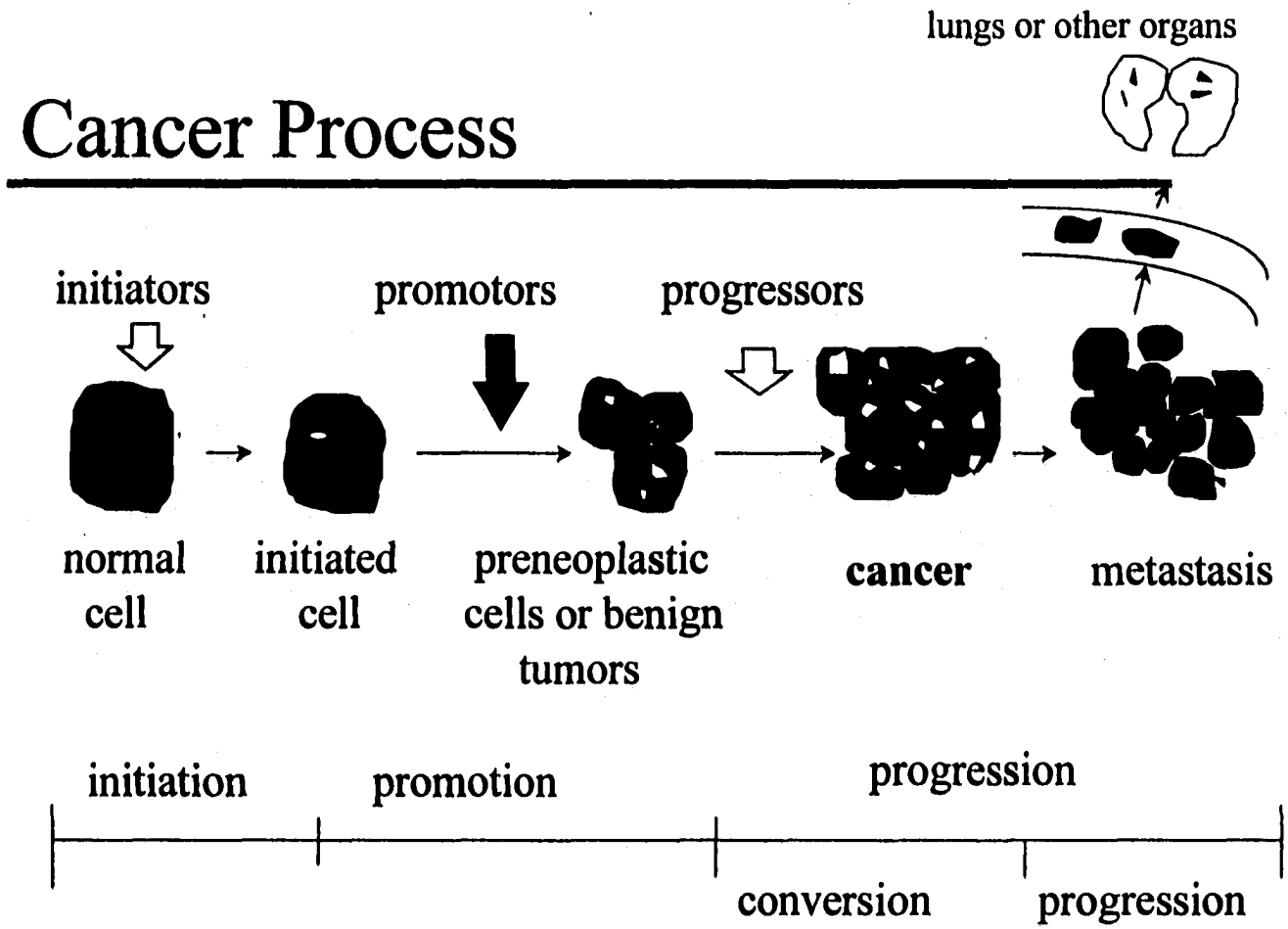

Figure 2.4 Mechanism of carcinogenesis
(adapted from Tanaka, 1997)

Organization (FAO) has estimated that twenty-five per cent of the world's food crops are affected by mycotoxins every year (FAO, 1996). Aflatoxin B_1, the most potent of the mycotoxins, is a food-borne fungal secondary metabolite produced by the *Aspergillus flavus* and *A. parasiticus*. It is highly toxic, carcinogenic and mutagenic and is involved in animal toxicosis and primary liver cancer (PLC) in humans.

Diet is a complex mixture of chemical entities. Recent research indicates that in addition to macro- and micro-nutrients, and mutagens and carcinogens, the human diet contains a number of antimutagens and anticarcinogens (Stich, 1991; Stavric, 1994). Antimutagenic agents are natural or synthetic compounds and which are able to lower or abolish the genotoxic effects of mutagenic and carcinogenic factors by diverse modes of action. This is in close agreement with epidemiological studies that have found a negative association between cancer and fiber-containing foods, such as fresh fruits and vegetables (Archer, 1988; Birt and Bresnick, 1991). Epidemiological data demonstrate correlations between dietary factors and the incidence of large intestinal cancer (LIC). Certain fiber-rich diets are associated with a lower risk of LIC; those high fiber diets are also rich in phytic acid (Ullah and Shamsuddin, 1990).

Since time immemorial the use of terrestrial plants in the armamentarium of human therapeutics has been recorded. It is well known that fruits and vegetables contain a marked amount of antimutagens/anticarcinogens, in particular, flavonoids (Sugimura *et al.*, 1996). Ellagic acid, a naturally occurring phenolic compound in grapes, strawberries, raspberries, black currants and

walnuts, inhibited the mutagenecity of AFB_1 in Ame's test (Loarca-Pena *et al.*,1996). Chlorophyllin, a food grade derivative of chlorophyll in green plants, has shown dose-dependent antimutagenic effects against benzo(a)pyrene, AFB_1-DNA adducts, and 2-aminoantherene in a rainbow trout model (Brienholt *et al.*, 1995). Some constituents (indole-3-carbinol) of cruciferous vegetables have shown protective effects against AFB_1-induced hepatocarcinogenesis in the same rainbow trout model (Dashwood *et al.*, 1988) and in the Ames *Salmonella* mutagenecity assay (Takahashi *et al.*, 1995). Antimutagenic effects have also been observed in green, fermented (black tea), and semi-fermented (oolong tea) tea extracts towards benzo(a)pyrene (B[a]P and AFB_1 also using the Ames *Salmonella* assay (Yen and Chen, 1994). Whole grain or bran milling fractions of rye, wheat and barley contain alkylresorcinols, which have antioxidant activity and this decreases the mutagenic effects of promutagens (B[A]P and 2-aminofluorene[2-AF]) and direct-acting mutagens (methanesulfonate and daunorubicin) in a dose-dependent manner (Gasiorowski *et al.*, 1996). Organosulfur compounds (methyl propyl-disulfide and propylene sulfide) separated from allium vegetables such as garlic and onion, have also been demonstrated for their modifying effects on hepatocarcinogens in rats (Yin and Cheng, 1998).

The use of synthetic phenolic antioxidants, i.e., butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT), has been shown to inhibit the carcinogenicity of a variety of carcinogens in different tissues of mice and rats (Williams, 1993; Wattenberg, 1985). The inhibition of AFB_1 hepato-

carcinogenesis in male Fischer-344 rats was found to be dose-dependent and a level of 125ppm of either antioxidant inhibited the initiation of hepato-carcinogenesis (Williams and Iatropoulos, 1996).

The search for antimutagenic agents is important since mutagenic and carcinogenic factors are omnipresent in the human environment and elimination of all of them seems not a viable proposition and it is important to discover naturally-occurring or synthetic compounds, which can suppress or prevent the process of carcinogenesis. Consumers, however, are concerned about the safety of synthetic food antioxidants. This concern has produced an increased interest in the use of natural antioxidants in the preservation of foods. The best candidates appear to be natural diet components, taken in sufficient concentration during regular daily meals. It is, therefore, little wonder that anti-mutagens and anti-carcinogens will continue to be isolated from foodstuffs of plant origin. Although research has focused on identifying the unknown factors in such agricultural commodities, there still is a thrust to re-evaluate those components, which have already shown antimutagenicity in various *in vivo* and *in vitro* studies (Pezzuto, 1997).

F. Food and Chemopreventors/Anti-mutagens

Foods contain mutagens and carcinogens, some of which occur naturally and others could be introduced during the preparation of foods for consumption (Pariza *et al.*, 1990). Some of the examples incude naturally occurring mycotoxins from *Aspergillus* and *Fusarium* species, heterocyclic amines in fried meats, polycyclic aromatic hydrocarbons (PAHs) in cooked/heated foods,

nitrosamines in fermented foods, and pyrrolizidine alkaloids (PAs) from plants (Sugimura, 1996). Most of these mutagens are metabolized by cytochrome P-450 enzymes, resulting in the formation of reactive compounds to produce DNA adducts. It has been observed that human cancers are not produced by a single carcinogenic agent but rather are the result of many carcinogenic compounds acting in concert and each acting at very low exposure levels (Sugimura *et al.*, 1996).

Search for antimutagenic compounds is important. The human diet contains a number of antimutagens and anticarcinogens (Stich, 1991; Birt and Bresnik, 1991). This is in close agreement with epidemiological studies that have found negative associations between cancer and fiber-containing foods, such as fresh fruits, and vegetables. The role of dietary factors in the prevention of major chronic diseases, cancer in particular, is under intensive investigation by many laboratories around the world. However, at present it is difficult to comprehend the role of the antimutagens/anticarcinogens in foods in terms of reducing cancer incidence. There are many unknown constituents and factors in foods, besides those mentioned above, that could enhance or reduce the possibilities for developing cancer (Stavric, 1994). Although several reviews have been published in this regard, the mechanisms of the inhibition of carcinogenicity by dietary components are still not clearly defined (Stavric, 1994).

It has been observed that a number of regular food components, which belonging to different chemical groups, possess cancer preventive properties and

other beneficial outcomes related to other diseases. These chemicals, therefore, are frequently known as chemopreventors (CPs) or phytochemicals. Chemically, they belong to a variety of different classes of chemicals and a large number of CPs are normal food components. The most thoroughly investigated CPs in foods are fiber, polyphenolic compounds, flavonoids, epigallocatechin gallate and other catechins, conjugated isomers of linoleic acid, soybean proteins, isoflavones, vitamins (A, B, C, E), tocopherols and tocotrienols, calcium, selenium, chlorophylline, sesaminol, coumarins, uric acid, indoles, thyiocynates, and protease inhibitors (Stavric, 1994). Over 25 different classes of chemicals have been found to possess antimutagenic/anticarcinogenic capacities. They are found in all categories of foods, with fruits and vegetables being the main source. Table 2.1 summerizes recent developments in the search of already identified and possibly some unidentified antimutagenic/ anticarcinogenic components and their beneficial effects in the prevention of cancer and other chronic diseases. The amount of CPs in different categories of foods can, however, vary considerably. It has been reported that even the same type of food products, obtained from different regions, may contain different levels of a particular CP (Wattenberg, 1990).

The anticarcinogenic and antimutagenic mechanisms of CPs appear to be complex. Although the beneficial activity of CPs depends on many unrelated factors and conditions, this effect could be the result of a single event or the simultaneous action of several factors acting in concert.

Table 2.1 Categories of foods and chemopreventors

Type of Food	Chemopreventors
Fruits	Vitamins, fiber, flavonoids, minerals, polyphenolic acids.
Vegetables	Vitamins, fiber, flavonoids, chlorophyll, carotenes, metals, phytic acid, isothyiocyanates.
Cereals	Fiber, tocopherols, phytic acid, minerals.
Nuts, Beans, Grains	Polyphenolics, fiber, vitamin E, phytic acid.
Meat, Fish, Poultry	Vitamins (A, E), linoleic acid.
Fat/Oil	Fatty acids, vitamin (A, D, E, K)
Spices	Coumarins, curcumin, sesaminol.
Tea, Coffee, Wine	Flavonoids, plant polyphenols, epigallocatechin.

Source: Adapted from Stavric, 1994.

Wattenberg (1990) has therefore suggested that the composition of the diet can be an important factor in determining the response of the individuals to carcinogenic agents to which they are exposed. In general, it appears that many of CPs are antioxidants, and as such, they may scavenge free radicals, formed during the preparation of food, or by the biological processes in the body. As free radicals damage lipids, proteins, cell membranes and DNA, their removal can prevent certain chronic diseases, particularly cancer or atherosclerosis (Stavric, 1994). These factors counteract the mutagenic effect to celular DNA by either blocking their access to potential mutagens or reacting directly with

mutagens (desmutagens) and/or by suppressing cellular mutagenesis by means of enhancement of repair mechanisms and suppressing errors in the DNA repair processes (bio-antimutagens).

1. Chemopreventors and Their Mechanisms of Action

The mechanisms of action of the CPs can be separated into two main categories and sub-groups. The *extracellular* mode of action includes: the inhibition of mutagens/carcinogens during preparation of foods and the effects in the intestinal transit phase(s), and *intracellularly*: by scavenging reactive oxygen species, inhibiting metabolic activation, reducing the detrimental effect of pro-carcinogens on DNA, and by enhancing and/or inhibiting the enzyme(s) involved in detoxification and formation of mutagens/carcinogens as required. Any of these actions, either alone or in combination, will reduce the hazardous activity of the mutagen/carcinogen in the body.

Although the mechanisms appear to be very heterogeneous, the antioxidative characteristics of CPs seem to play the most significant part in their protective activity (Borek, 1990). Not all CPs will be equally beneficial but conversely, may exhibit several different mechanisms of action simultaneously. Some of them can act both extracellularly, by reducing the formation and bioavailability of carcinogenic species, and also intracellularly, by influencing a particular enzymatic system (Stavric, 1994). Some examples of CPs in common foods and their proposed mechanisms of action are discussed below by compound.

a. Plant polyphenols

It is generally assumed that the active dietary constituents contributing to protective effects toward cancer and cardiovascular diseases are the antioxidant nutrients, but more recent work is highlighting the additional role of the polyphenolic components of the higher plants. Flavonoids occur naturally in plant foods and are a common component of our diet. They consist mainly of anthocyanidins, flavonols, flavones, flavonones, isoflavones, and catechins (Hertog *et al.*, 1993). Important dietary sources of flavonoids are vegetables, fruits, and beverages, the latter accounting for at least 25-30% of the total daily flavonoid intake. They generally occur as *O*-glycosides with sugars bound at the C3 position and have been found to have the capability of altering the levels of phase I and phase II enzymes. Some have shown a selectivity on either the induction of specific metabolizing enzymes or induction of these enzymes in specific tissues (Smith and Yang, 1994). Recent studies have reported that polyphenols also scavenge superoxide and hydroxyl radicals, reduce lipid peroxyl radicals, and consequently inhibit lipid peroxidation (Salah *et al.*, 1995).

Flavonoids demonstrated a wide range of biochemical and pharmacological effects, including anti-inflammatory and anti-allergic effects (Middleton and Kandaswami, 1992). Food-derived flavonoids such as ellagic acid, chlorogenic acid, rutin, quercetin, kaempferol, and myricetin have been shown to inhibit carcinogen-induced tumors in laboratory animals (Deschner *et al.*, 1991). Several studies have strongly suggested that ellagic acid (EA), a natural polyphenol abundant in many fruits and vegetables such as grapes,

strawberries, nuts and other foods, could be effective in preventing the development of cancer induced by tobacco carcinogens (Castonguay *et al.*, 1990). Chlorogenic acid (CA), a normal component of peaches, blueberries, and coffee beans, along with EA has found to inhibit carcinogenesis in several animal studies. Similarly, quercitin (Q) and rutin (RU), both present in fruits and vegetables, are consumed by North Americans at about 1g/day. It appears that these two polyphenols inhibit colonic neoplasia induced by azoxymethanol, a known carcinogen (Deschner, 1991). Catechin, a flavan-type polyphenol, found in sorghum grain, fava beans, and green tea was found to be both an antimutagen and an anticarcinogen. It inhibits the mutagenicity of B[a]P and dimethylbenzo[a]anthracene (DMBA) in the *Salmonella* microsomal mutagenicity assay, suppresses nitrosation of methylurea and reduces B[a]P-induced forestomach tumors in mice (Nagabhushan, 1990).

Tea, originating from China, is one of the world's oldest prepared beverages. It is consumed extensively throughout the world in the form of different tea extracts including hot and cold infusions. Quercithin, which is a major flavonoid in tea (*Camelia sinensis*) including black and green tea, has been demonstrated to inhibit oxidation and cytotoxicity of low density lipoproteins (LDL) *in vitro* (Negre-Salvagyre and Salvagyre, 1992) and would decrease cancer and cardiovascular diseases in humans. Different teas have been analyzed for their antimutagenic and anticarcinogenic effects (Hertog, *et al.*, 1993; Yen, *et al.*, 1994). Tea extracts including black (fermented), oolong and pouchong (semi-fermented), and green (non-fermented) teas were compared for

their toxicity and mutagenicity in the *Salmonella*/microsomal mutagenicity assay in presence of different toxic and mutagenic chemicals (Yen *et al.*, 1993). All teas were found to inhibit mutagenicity especially the semi-fermented teas (Oolong and pouchong) where up to a 90% reduction in mutagenicity was observed. Modulation of aflatoxin B_1 metabolism has been suggested as a possible mechanism of action for the anti-mutagenic properties of green teas. Green or semi-fermented tea(s) could possibly be beneficial to humans for the purpose of mutation chemoprevention.

The major beneficial component in green tea has been identified as polyphenol (-) epigallocatechin gallate (EGCG), a compound that possesses antioxidant properties. This compound appears only in smaller quantities in black tea due to their oxidation during the fermentation process. The EGCG or green tea extract has been tested extensively and found to reduce different types of spontaneous or chemically induced tumors in laboratory animal studies, for example tumors of the liver, stomach, skin, lung, and esophagus (Huang, *et al.*, 1992). Chinese and Sri-Lankan (Ceylon) teas were tested positively in preventing the formation of mutagenic nitrosated compounds found in fish prepared for consumption. The beneficial effects were attributed to the presence of catechin, epicatechinic derivatives, quercitin, kaempferol, and myricetin (Klaunig, 1992). The same types of phenolic compounds were found in red wine, and it has been suggested that these plant phenolics were responsible for the anti-mutagenic activity of red wine.

Although brewed coffee has a weak mutagenicity in the Ames *Salmonella*/microsomal mutagenicity test, it also contains antimutagenic /anticarcinogenic compounds such as chlorogenic acid (CA), which can be found in instant coffee powder in amounts between 3 and 12% (Clifford, 1985). Natural antioxidents from wine and ciders have attracted considerable interest. Since for the production of wine, the pressed juice is made from grapes, berries and other fruits, the presence of flavonoids and other phenolic compounds have been attributed to their antioxidant properties.

b. Vitamins

For millions of health-conscious Americans, antioxidant vitamins are a way of life. Revered for their power to subdue free radicals, vitamin supplements have sold wildly in recent years. In 1993 alone, store sales of vitamin E supplements grew by 39% ($123 million), beta-carotene ($22 million) and vitamin C ($117 million) (Bagely, 1994). The evidence linking vitamins to better health is considerable. No one denies that folic acid (one of the B vitamins) can help prevent birth defects, that vitamin C can reduce the risk of mouth, throat and stomach cancers, that vitamin D may help prevent breast, bowel and prostate cancers, and that vitamin E can reduce the risk of heart disease.

Comprehensive reviews on the role of vitamins as chemopreventors have been published recently (Berger *et al.*, 1991; Merrill *et al.*, 1991). Some studies have supported the hypothesis that there may be an inverse relationship between the dietary intake of ascorbic acid, beta-carotene, alpha-tocopherol and the risk

of human cancer, whereas some other studies have found no such relationship (Prasad, 1990). Certain vitamins induce cell differentiation in well-established cancer cells in culture and also reduce the action of tumor promoters and initiators (Prasad, 1990). Although the mechanisms of these actions are still not well understood, the potential of vitamin supplements to protect against cancer should be explored in future human intervention studies, some of which are already in progress.

Vitamin A: Retinol (vitamin A) is another vitamin with modifying effects on the malignant transformation of cells induced by chemicals, viruses, and radiation (Stavric, 1994). In laboratory animals, it has been demonstrated that retinoids can prevent cancer of skin, lung, urinary bladder, breast, esophagus and stomach. Beta-carotene, which is the most important of the provitamins A, is widely distributed in the plant and animal kingdom and has scavenging capacity for free radicals (Stavric, 1994).

Vitamin B: Riboflavin (vitamin B complex) reduced the mutagenicity of B[a]P in the presence of a homogenate of rat liver enzymes (S-9) but not of the ultimate B[a]P metabolites (Ejchart *et al.*,1990). It appears that the antimutagenicity of riboflavin originates from an interaction between riboflavin and one or more enzymes in the liver S-9 fraction.

Vitamin C: Epidemiological evidence suggests that vitamin C (or other components of fruits) is protective against several types of cancer in humans. The multiple and complex effects of vitamin C on a variety of biological activities, especially its effects on the development of cancer, are still not well

understood. Many of the beneficial biological effects vitamin C appears to be related are to its chemical properties and not to its role as a vitamin. For example, vitamin C acts effectively as a scavenger of nitrite in the stomach, thereby preventing the formation of carcinogenic nitrosamine species and free radicals found during the processing of foods or in metabolic processes (Borek, 1990). Plasma devoid of vitamin C, and without other antioxidants, is extremely vulnerable to oxidative stress and is susceptible to peroxidative damage of lipids. The role of vitamin C in cancer, including its effects on delaying onset and growth of tumors, prolonging survival, reducing toxicity as a result of chemothrapy treatment, and the efficacy of concomitant use of vitamin C has been reported extensively.

Vitamin E: Different isomers of vitamin E (tocopherols and tocotrienols) appear to have a multitude of functions. They are most commonly found in whole cereal grains, bran milling fraction, nuts, and vegetables. It is the major antioxidant in the lipid phase of cells and protects polyunsaturated fatty acids against peroxidation, frequently called oxidative stress. Consequently, it reduces free radical formation, which in turn may result in a decrease of carcinogenesis and other degenerative diseases. It also has some inhibitory effects on proteases as protease inhibitors are assumed to protect against radiation damage, as inhibitors of mouse skin tumorigenesis and in prevention of colon cancer (Stavric, 1994).

c. Soybean effect

The rate of breast cancer is lower in oriental countries than the west. A large-scale consumption of soybean in oriental countries compared with limited use of this commodity in the Western diet, may have a role in preventing breast cancer. It appears that isoflavones in soybean are the active antitumorigenic components (Messina and Barnes, 1991; Messina, 1991). Some other studies have suggested that phytoestrogens in soy products may provide the protective effects against breast cancer (Lee *et al.,* 1991). In addition, CA, a polyphenolic antioxidant in soy protein concentrates, may also decrease the mutagen formation. Soybeans also contain genistein, which may keep tiny tumors from getting contacted to capillaries that carry oxygen and nutrition, and without these supply lines, the tumor never grows and/or metastasizes. In addition to these compounds, phytates, a major constituent in soybean, has been reported to inhibit aflatoxin biosynthesis (Hesseltine, 1963).

d. Fiber and phytic acid

The role of plant-derived fibers in the protection of human mutagenesis and carcinogenesis has been studied extensively. The major dietary fiber comes from cereal grains and bran milling fractions. It has been assumed that fiber dilutes carcinogens through increasing the bulk of stool and thus reduces the transition time of carcinogens in the stomach and intestine, but contrary to this fact, an additional component, phytic acid, has been found besides fiber. Several studies in animals have supported that phytates, and not only fiber (as a helping component) have the chemopreventive effects against large intestine cancer

(Eaton and Graf, 1985). Phytate, a major constituent in soybean, has been reported to inhibit aflatoxin biosynthesis (Hesseltine, 1963). The role of phytic acid will be discussed in a separate section.

Cereal grains are not only rich in the above-mentioned compounds but besides fiber and non-starch polysaccharides, bran milling fraction also contains a significant amount of alkylresorcinols (AR). Although the biological role of alkylresorcinols has not been established, it has shown antioxidant properties due to its phenolic nature (Gasiorowski *et al.*, 1996). It has been demonstrated to markedly decrease the mutagenicity of standard indirect-acting mutagens such as B[a]P and 2-AF, and direct-acting mutagen, daunorubicin, in the Ames test (Gasiorowski *et al.*, 1996).

e. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) contain two or more unsaturated double bonds. In mammalian systems, PUFAs can be classified into two broad categories termed "essential" and "non-essential" fatty acids (Bruckner, 1992). Essential PUFAs are considered by most to include fatty acids of both the n-6 and n-3 fatty acid families, for example, all-cis-18:2n6 (i.e., linoleic acid) and all-cis-18:3n3 (i.e., linolenic acid). These essential linoleic acid must be provided in the diet because they cannot be synthesized from simple carbon precursors in a mammalian cellular system (Bruckner, 1992). They have a structural as well as biochemical function and are integral components of phospholipids. Dietary linoleic and linolenic acids are metabolized by desaturation and chain elongation. The presence of linoleic and linolenic acids

in foods have been regarded as beneficial. Their beneficial role in cardiovascular disease, cancer, inflammation, renal disease and diabetes mellitus have been reported extensively in the literature (Bruckner, 1992; Keys *et al.*, 1980). It was reported that the consumption of increased amount of dietary n-3 fatty acids contibuted to the low incidences of ischemic heart disease in Greenland Eskimos (Dyerberg and Bang, 1979). Several reports have also suggested that not all fat types promote tumorogenesis and fatty acid composition plays an important role in cancer promotion (Carroll, 1983). It was observed that tumorogenesis was inhibited by linoleic acid *in vivo*.

The anti-mutagenic properties of unsaturated fatty acids have been reported both *in vivo* and *in vitro* (Aikawa, 1988; Aikawa and Komatsu, 1987; Fischer *et al.*, 1996; Huang *et al.*, 1997). It has been reported that linoleic and oleic acids inhibited the irradiation-induced mutagenesis in *Escherichia coli* (Aikawa and Komatsu, 1987). It was also reported that linoleic acid was anti-mutagenic in Ames *Salmonella* mutagenicity assay against 2-aminofluorene (Aikawa, 1988). Recently cow's milk components have been reported to be anticarcinogenic (Fischer *et al.*, 1997). Conjugated linoleic acid was identified in playing a role in the inhibition of mammary tumorogenesis *in vivo* (Fischer *et al.*, 1997), and colorectal, lung and skin carcinogenesis in cancer cell lines (Aikawa, 1988).

The role of linoleic acid in the inhibition of aflatoxin biosynthesis from *Aspergillus* spp has also been reported (Burow *et al.*, 1997; Croft *et al.*, 1993; Hamid and Smith, 1987; Montes-Belmont and Carvajal, 1998). Furthermore,

several reports have also observed the presence of linoleic acid-like compounds present in corn and found it anti-mutagenic against intrinsic AFB_1 (Burgos-Hernandez, 1998; Huang *et al.*, 1997).

f. Selenium

Selenium is consumed in the form of selenite and selenate, and is available through drinking water and some grains, beans, and nuts. The chemopreventive effects of selenium are attributed to its antioxidant properties and its involvement with the enzyme glutathione peroxidase. It has been reported in various studies that even microgram quantities of selenium reduces the risk of skin, colon, liver and breast cancer in animal studies (Stavric, 1994).

g. Miscellaneous chemopreventors

Allium vegetables such as garlic and onions are largely consumed in China, Indian sub-continent and Middle-eastern countries. They contain allylic sulfides. Their increased consumption has been shown to significantly reduce the risk of gastric cancer. The beneficial effect of garlic extract in animal studies has been found to inhibit oral cancer (Stavric, 1994; Hrelia *et al.*, 1996).

Epidemiological and experimental evidence indicates that consumption of a diet high in cruciferous vegetables, such as broccoli, cabbage or brussel sprouts, is associated with reduction in the incidents of cancer. The active components, apparantly are dithiolthiones, isothiocyanate, and sulphoraphane. The protective effects of dithiolthiones and sulphoraphane are associated with a number of biochemical changes, the most notably being the increases in the levels of reduced glutathione (GSH). Broccoli is a bonanza vein of CPs. The

anticarcinogenic action of broccoli appears to be due to the induction of detoxification enzymes by sulphoraphane (Zhang *et al.*, 1992). Other components in cruciferous vegetables are dietary fiber, polyphenols, vitamin C and chlorophylline.

Chlorophylline, a derivative salt of chlorophyll, possesses the ability to inhibit the mutagenic activity of a variety of complex environmental mixtures, including food mutagens formed during the frying of meat. It either acts as a scavenger of free radicals or interacts with the active parts of the mutagenic compunds. Chlorophylline was found antimutagenic in the Ames *Salmonella* microsomal mutagenicity test against aflatoxin B_1 and 3-amino-1-methyl-5H-pyrido indol (Trp-P-2) (Breinhold *et al.*, 1991).

Tomatoes are rich in coumaric acid and chlorogenic acid. Strawberries, pineapple and green peppers are also rich in these acids. During digestion the body routinely makes nitrosamine, both of these acids work by disrupting the chemical weeding of nitric oxide and components of proteins called amines. Hot chilli peppers are rich in capsacin, which keeps carinogens like those in cigarette smoke from binding to DNA, where they can trigger the changes that lead to lung and other cancers.

h. Therapeutic plants

From time immemorial, the plant components have played a dominant therapeutic role in the treatment of human ailments including cancer. Four Chinese medicinal plants, *Oldenlandia diffusa, Scutellaia barbata, Astragalus memberanaceous,* and *Lingustrum lucidum,* with a long history of indigenous

use as antitumor agents or as adjuncts have been demonstrated to be active in the Ames assay by reducing metabolic activation by P450 isoenzymes against aflatoxin B_1 (Mitscher *et al.*, 1996; Hrelia *et al.*, 1996). In Western civilization, terrestrial plants have also continued to play an important role in health care. It has been summarized recently that approximately 120 available drugs are plant-derived products and a large number of therapeutic activities are mediated by these drugs. There is a host of drugs currently in use which are still obtained from plants from which they are synthesized (Pezzuto, 1997). Some of the examples include steroids, Cardiotonic glycosides, antichlolinergics (belladonna-type tropane alkaloids), analgesics and antitussives (opium alkaloids), antimalarial (*Cinchona* alkaloids), antigout (colchicine), anesthetic (cocaine), skeletal muscle relaxant (tubocurarine), and anticancer agents (from a variety of plant components). The antitumor agents paclitaxel (Taxol), vincristine (oncovin), and podophyllotoxin are naturally found and obtained from *Taxux brevifolia, Catharanthus roseus and Podophyllum peltatum,* respectively (Pezzuto, 1997). There is a continual search and research for more and more antimutagenic /anticarcinogenic compounds of plant origin since a natural component is considered to be the best counteracting agent against carcinogens/mutagens.

In spite of their properties, the chemopreventors or phytochemicals are not omnipotent. Even lifelong vegetarians get cancer. It has been proven that only a few vitamins would not undo the damage caused by heavy smoking. Although both vitamins C and E are antioxidants, its booster claim that they

fight aging and cancer, proved no match in a cohort study in Finland. The men on β-carotene got 18 percent more lung cancers than those on placebos and those on vitamin E suffered 50 percent more fatal strokes. If there is a take-home message in all this, it is the old adage that eating a diet rich in fruits and vegetables prolongs a healthy life. Although no long term study in humans has shown that any particular phytochemical retards cancer, the lab results dovetail perfectly with about 200 studies linking diets rich in all sorts of fruits and vegetables with a lower risk of cancer. Everyone is looking for a magic pill, but it should be realized that taking a tablet or a fancy new CP, instead of the recommended five daily servings of fruits and vegetables, is nutritional madness.

G. Phytic Acid (*Inositol Hexaphosphate*, $InsP_6$)

1. Introduction

The seeds of certain plants may remain viable for 400 years (Erdman, 1979). The reasons for their remarkable longevity are largely unknown. These hardy properties are particularly puzzling considering that seeds contain a potentially reactive mixture of large amounts of highly unsaturated lipids, iron and oxygen. Clearly these elements within seeds must conspire to prevent the occurrence of extensive oxidative injury which would lower their germinability (Graf and Eaton, 1990). These partial protections against oxidative damage are attributed to the presence of natural antioxidants and phenolic compounds, and also are conferred by severe dehydration of seeds during storage, which decreases the kinetic mobility of these reactants and catalysts. Phytic acid (PA),

or *myo*-inositol hexaphosphate ($InsP_6$), a major phosphorus storage compound of most seeds and cereal grains, contribute about 1 to 7 % of their dry weight (Zhou and Erdman, 1995). Phytic acid also referred to as phytates, first identified in 1855, represent a complex of naturally occurring compounds that can significantly influence the functional and nutritional properties of foods. Their presence, chemical structure, health implications and possible role in the human diet are addressed in detail.

2. Occurrence

Phytic acid (PA), or *myo*-inositol hexaphosphate ($InsP_6$), is a major phosphorus storage compound of most seeds and cereal grains (Zhou and Erdman, 1995). It is a naturally occurring compound formed during the maturation of seeds and cereal grains. In the seeds of legumes, it accounts for about 70% of the phosphate content and is structurally integrated with the protein bodies as phytin. Phytic acid usually occurs as mixed calcium-magnesium-potassium salt of inositol (Erdman, 1979) in discrete regions of the seeds, such as the aleurone layer of wheat and rice (Lott, 1984). O'dell, (1972) demonstrated that, in corn, 90% of PA is concentrated in the germ portion compared to the endosperm and hull portions.

3. Structure and Chemical Properties

PA is the most abundant form of phosphorus in plants (Figure.2.5). During food processing and digestion, it can be partially dephosphorylated to produce degradation products, i.e., penta-, tetra-, and tri- phosphates by the action of endogenous enzymes called phytases, which are found in most of the

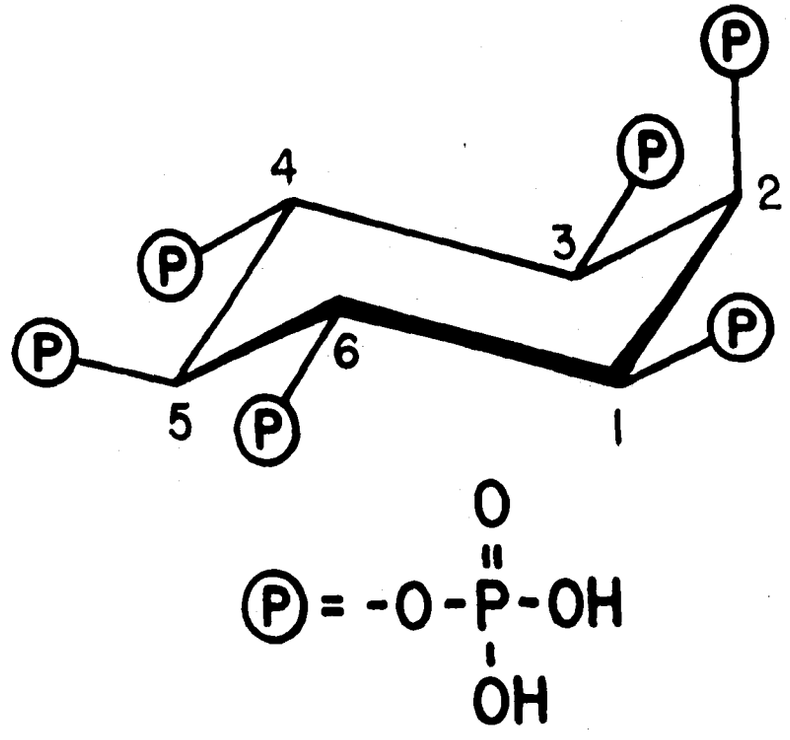

Figure 2.5 Chemical structure of phytic acid (*Inositol Hexaphosphate*)
(adapted from Graf and Eaton, 1990)

phytic acid-containing seeds in higher plants (Sandberg *et al.*, 1987). Both acid hydrolysis and enzymatic treatments with phytase result in a mixture of *myo*-inositol, inorganic phosphate and myo-inositol mono-, di-, tri-, tetra-, penta-, and hexaphosphate. During seed germination phytase activity increases resulting in the hydrolysis of phytic acid and the release of phosphate and free myo-inositol for plant development. In the human gastro-intestinal tract, the hydrolysis of PA is carried out by the action of phytases from, dietary plant phytases, phytasesfrom bacterial flora in the gut, and intestinal mucosal phytases (Graf, 1986; Williams and Taylor, 1985). Other *neo-, chiro- and scyllo-* inositol hexaphosphates forms have been isolated from soils, where they exist as a mixture of these isomers (Reddy et al, 1982).

Since its discovery, the reputation of PA has had a roller coaster ride. It has been considered anti-nutrient due to its inhibitory effect on mineral bioavailability. Its strong chelation ability with multi-valent cations, especially di- and trivalent cations, to form cation-PA complexes (Vohra *et al.*, 1965), result in the insolubility of these complexes and are regarded as the major reason for the reduced bioavailability of PA mineral complexes. Minerals of concern in this regard would include zinc, iron, calcium, copper, and phosphorus. In particular PA induced zinc deficiency has been reported extensively in the literature (Erdman, 1979; Maga, 1982; Reddy *et al.*, 1982).

Recently, this chelation ability of PA with minerals has been reported beneficial towards reducing/lowering serum cholesterol and triglycerides (Jariwala *et al.*, 1988; Klavay, 1977) and suppressing iron-mediated oxidation

and decreasing colon-cancer risk (Heany and Weaver, 1991; Shamsuddin, 1992). It is also considered a natural antioxidant due to its inhibition of iron-catalyzed hydroxyl radical formation and lipid peroxidation (Empson *et al.*, 1991; Graf *et al.*, 1987). Biochemists and cell biologists have been interested in the phosphorylation and dephosphorylation of phytic acid and how this might affect cellular functions. Lower inositol phosphates (InsP 1-4) are recognized as intra-cellular messengers. The second messenger role of inositol (1, 4, 5) tri-phostphate ($InsP_3$) in bringing about a host of cellular functions including mytosis via mobilizing intracellular calcium (Ca^{2+}) is well-recognized; $InsP_4$ has also been shown to induce Ca^{2+} sequestration. Higher forms of InsP (tetra-, penta- and hexa-) are also abundant and represent the bulk of PA content in mammalian cells. Why should there be an intra-cellular abundance of these compounds that are presumed toxic by nutritionists or inert at best by biochemists? (Shamsuddin, 1995).

Phytic acid also forms strong electrostatic linkages with amino acyl residues at low pH and thereby precipitates most protein below pH 5.0 (Graf and Eaton, 1990). By virtue of binding to proteins, it has been found to inhibit polyphenol oxidase (Graf *et al.*, 1987), α-amylase (Thompson, 1994), alcohol dehdrogenase (Altschuler and Schwartz, 1984), trypsin (Singh and Krikorian, 1982), and other enzymes. PA also has a high affinity for 2, 3-diphosphoglycerate sites in hemoglobin (Eaton and Graf, 1990), and results in the modification of heme iron-O_2 interaction, which facilitates dissociation of

oxygen from hemoglobin. Therefore, phytic acid may prove useful in the treatment of ischemia, hemolytic anemia, pulmonary insufficiency and hypererythropoiesis by improving O_2 transport capabilities, if it is incorporated in erythrocytes (Nicolau *et al.*, 1986; Weiner and Franco, 1986).

4. Mineral Bioavailability and Nutritional Considerations

Numerous studies have led to the conclusion that PA and its derivatives can bind to essential dietary minerals, thus making them unavailable or only partially available for absorption. The most commonly studied minerals are calcium, iron and zinc.

a. Calcium

PA-Ca complex is considered to be the major contributor that reduces the bioavailability of other minerals i.e., Zn and Fe. Zn and Fe can form even less soluble complexes than the PA-Ca complex. The degree of phosphorylation plays an important role in inhibition as less phophorylation renders no effect and higher phosphorylation (5-6 phosphate) significantly inhibits the solubility. It is suggested that people with higher Ca requirements (e.g., children and older people) or those with low calcium intakes should decrease PA consumption.

b. Iron

McCance *et al.*, 1942 was the first to report that phytic acid inhibited iron absorption. Since then many studies have been carried out in animals and humans showing that PA has a very strong inhibitory effect on iron absorption (Brune *et al.*, 1992; Hallberg *et al.*, 1989; Tuntawiroon *et al.*, 1990), and which have been due only to PA and other inositol phosphates in bran and not due to

fiber and other constituents. The studies also reviewed that the removal of PA in bran by endogenous phytase significantly increased iron absorption (Hallberg *et al.*, 1987). Furthermore, PA reduction in soybean and soy foods by fermentation has been shown to result in the prevention of iron deficiency, and subsequently anemia in children (Qin *et al.*, 1989). However, a few studies have failed to show the inhibitory effect on iron absorption (Hunter, 1981). Heat treatment had been found to improve iron retention due to increased PA degradation (Erdman and Fordyce, 1989), which from a nutritional standpoint would be unacceptable due to amino acid destruction. The question of adaptation to a high phytate diet has been examined by Brune *et al.*, 1992. It has been suggested that an iron balance situation in subjects with high PA consumption can only be satisfactory when the diet also contains sufficient amounts of food components counteracting the inhibition of PA, such as ascorbic acid.

It can be concluded that PA is the major inhibitor of dietary iron bioavailability and that its removal significantly increases iron availability. For people with high iron status, increased dietary PA intake may be beneficial because high iron storage in the body has been associated with increased risk of ischemic heart disease, probably due to the role of iron in mediating free radical formation and subsequent lipid peroxidation (Sullivan, 1992; Sullivan, 1981; Salonen *et al* ., 1992). Conversely, for a population on a high PA diet and having low iron status, it would be wise to increase the consumption of foods containing high levels of organic acids such as ascorbic acid, to counteract the inhibitory effect of PA on iron absorption.

c. Zinc

Zinc (Zn) has been the most studied component in connection with dietary role of phytic acid. The results from human and animal studies have demonstrated that PA contained foods reduce Zn bioavailability (Davies and Olpin, 1979; Erdman, 1981; Erdman *et al.*, 1980; Forbes *et al.*, 1979; Forbes *et al.*, 1984; Lonnerdal *et al.*, 1984; Oberleas *et al.*, 1962; Oberleas *et al.*, 1966; O'Dell and Savage, 1966; Stuart *et al.*, 1986; Zhou *et al.*, 1992). The PA to Zn molar ratio was suggested as a parameter to predict the zinc bioavailability. The calcium content of a diet is of vital importance due to the negative impact of PA on Zn bioavailability. The addition of Ca to a phytate-supplemented diet further reduces utilization of dietary zinc (Oberleas *et al.*, 1962). Calcium exaggerates the inhibitory effect of PA on Zn bioavailability by forming insoluble calcium-PA-zinc complexes. Most of the attention in this area has turned towards soy products since their use in food systems is expanding. Soy based foods contain high amounts of minerals, especially iron, zinc, copper, calcium etc. along with a high amount of phytic acid and phytases. It was found that the bioavailability of zinc did not get affected because most of the bound Zn was liberated due to the action of phytase on phytate during fermentation.

The inhibitory effect of PA on Zn is considered to have some advantages due to the reduction of the antagonizing effect of Zn on copper absorption. It is thought that excess Zn results in decreased copper absorption by competing for common carrier systems that facilitate Zn and Cu absorption from the small intestine. When dietary PA content is increased, it preferentially binds Zn rather

than Cu. An elevated Zn to Cu ratio is an index that has been associated with hyperlipidemia (Klavey, 1977; Klavey, 1975; Jariwala *et al.*, 1990). It has been recommended to reduce PA contents in diets in those areas of the world where high prevalence of marginal zinc deficiency in the human populations occur.

5. Potential Positive Roles of Phytic Acid

In view of the results above, the evidence seems strong enough to consider PA as an antinutrient due to its adverse effects on mineral absorption. In the last few years, however, some novel metabolic effects of PA have been recognized. PA has been shown to function as a hypocholesterolemic agent, to prevent renal stone formation, suppress iron dependent oxidation (Sharma, 1986), and as an antioxidant (Empson *et al.*, 1991). Various animal studies and retrospective human epidemiological studies also suggest possible protective effects of PA on heart disease by controlling hypocholesterolemia and atherosclerosis in man (Sharma, 1986) and protecting an ischemic heart from reperfusion in rodents (Rao *et al.*, 1991). The most studied aspect has been colon cancer, and PA has been attributed to reducing the cancer mechanism in the colon through its antioxidant property as an inhibitor of iron-mediated free radical formation.

a. Antioxidant properties

The antioxidant capability of phytic acid results from the fact that PA is a potent inhibitor of iron-catalyzed hydroxyl radical (.OH) formation by chelating free iron and then blocking the coordination site (Graf *et al.*, 1984). Many biological processes result in the formation of Fe^{2+} and subsequent .OH

formation resulting in lipid peroxidation. Fe^{2+} has been shown to cause the production of oxyradical and lipid peroxidation, whereas Fe^{3+} is relatively inert (Halliwell and Gutteridge, 1989). This recently developed hypothesis asserts that phytic acid maintains iron in the Fe^{3+} oxidation state by changing the redox potential and obstructs the generation of hydroxyl radical (.OH) and other activated oxygen species by occuping all the available iron coordination sites (Graf *et al.*, 1987; Graf, 1983). All of the antioxidant properties of PA derive from its relatively high binding affinity for iron (Graf and Eaton, 1990).

Various studies have shown that iron-induced oxidative damage can be reduced in food by the addition of small amounts of PA (Empson *et al.*, 1991; Champagne *et al.*, 1990; Tsuno *et al.*, 1986), suggesting a food preservative property for phytic acid. It also provides a significant protection against ascorbic acid degradation and slowing lipid peroxidation in chick meal (Empson *et al.*, 1991). Various animal studies suggest PA as a therapeutically useful agent due to its antioxidant properties. It has also been reported that iron-mediated peroxidation of erythrocyte membrane lipids can be suppressed by PA (Ko *et al.*, 1990; Ko and Godin, 1991).

b. Anticancer role

Colon cancer is one of the major causes of morbidity and mortality in Western society (Silverberg and Lubera, 1987). The finding that U.S. born Japanese have a four-fold higher incidence of colon cancer than Japanese residents in Japan strongly suggests that diet, rather than genetic factors, is a predominant contributor to the incidence of colon cancer (Wynder and

Hirayama, 1977). PA or *InsP6* is a higher constituent of soybean products, which has been suggested as an important factor in the low colon, breast and prostate cancer in Japanese men and women. Phytic acid possesses significant antioxidant (Graf *et al.*, 1987, Graf and Eaton, 1990, and Oberleas, 1973) and antineoplastic (Shamsuddin, 1995) potential. The chemopreventive potential of phytic acid has been observed in the experimental models of colon (Ullah and Shamsuddin, 1990), liver (Hirose *et al.*, 1991) and mammary (Vucenik *et al.*, 1992) carcinogenesis.

Recent studies have shown that orally administered $InsP_6$ in drinking water inhibits experimental colon carcinogenesis in both F344-rats and CD1-mice (Shamsuddin and Ullah, 1988; Shamsuddin and Ullah, 1989; Shamsuddin *et al.*, 1989) and that it has beneficial effects on mammary carcinogenesis (Vucenick *et al.*, 1992). These results were confirmed by Pretlow *et al.* (1992) in a rat F344 model. Antitumor activity of phytic acid has also been suggested in murine transplanted and metastatic fibrosarcoma (Vucenick *et al.*, 1992). It has also demonstrated tumor inhibition properties in a rat fibrosarcoma tumor model (Jariwalla *et al.*, 1988) and hepatocellular carcinoma inhibition (Hirose *et al.*, 1991) in rats. Furthermore, Nielsen *et al.* (1987) demonstrated that phytic acid decreased colonic epithelial cell proliferation and also caused a reduction in cell proliferation in the mammary carcinogenesis model.

Regulation of cellular proliferation (Vucenick *et al.*, 1992), induction of differentiation in the cancer cells (Shamsuddin, 1995b), elevation of a host's defence against tumor via increased natural killer cell activity (Baten *et al.*,

1989), reduction in lipid peroxidation and chelation of different ions (Graf and Eaton, 1990) have been suggested as the probable mechanism(s) mediating the anti-neoplastic potential of phytic acid. The ability of phytate to bind with metals especially iron (Fe), may be responsible for phytic acid's antioxidant and anti-carcinogenic activity. By chelating Fe^{2+}, phytic acid inhibits Fe-induced free radical generation (Graf and Eaton, 1990). This is achieved by occupying all the available Fe^{2+} coordination sites, thus inhibiting .OH generation from the Fenton reaction. Subsequently, it limits the processes of lipid peroxidation and DNA damage by inhibiting free radical formation, which is thought to be involved in the etiology of certain cancers (Shamsuddin, 1995).

It has been hypothesised that $InsP_6$ (phytic acid) exerts its anti-neoplastic effect by reducing cell division, perhaps via the lower phosphorylated inositols, particularly inositol tri-phosphate ($InsP_3$) (Baten *et al.*, 1989; Graf and Eaton, 1985). Inositol phosphates are ubiquitous in most mammalian cells and are responsible for signal transduction and a host of cellular functions (Putney *et al.*, 1989; and Mitchell *et al.*, 1990).

c. Role in preventing heart disease

Heart disease is the leading cause of death in the world, yet it is low in Japan and developing countries. It has been suggested that PA, as a component of fiber, may play an important role in lowering the serum cholesterol level by influencing zinc and copper uptake (Klavey, 1973; Klavey, 1975a; Klavey, 1975b). The imbalance of a high zinc to copper ratio results in hyper-cholesterolemia, which is a factor in the etiology of coronary heart disease.

In animal studies, dietary PA supplementation has been shown to significantly lower serum cholesterol (Jariwalla *et al.*, 1990; Klavey, 1977) and triglycerides, accompanied by decreases in the serum zinc level and the zinc/copper ratio. Besides its effect on lowering the serum zinc/copper ratio, PA has also been reported in protecting against ischemic heart reperfusion injury by chelating iron and suppressing iron-induced free radical formation and subsequently the lipid peroxidation process (Rao *et al.*, 1991). Although the data involving human subjects are still lacking, the results from some epidemiological studies have shown some promising results against this leading cause of death in the western countries.

d. Role in preventing renal calculi

The increase of renal stone incidences throughout the world have been reported concomitant with the process of industrialization, especially in the developed world. It has been shown in epidemiological studies that there were substantial differences in renal stone incidents between white and black residents of South Africa. The major dietary difference is that, compared to the white population, blacks consumed large amounts of cereals and legumes as their staple, which contain high amounts of fiber and PA (Andersen, 1969; Modlin, 1967; Wise and Kark, 1961).

The process of stone formation depends on hydroxiapatite crystals, which function as nuclei for stone formation. There has been concern that high dietary calcium intake may result in increased urinary calcium excretion and a subsequent increase in renal stone incidence. On the contrary, a phytate-rich rice

bran diet has been reported to reduce significantly the intestinal absorption and urinary excretion of calcium when dietary calcium was high in a 3 year human intervention study (Modlin, 1980; Ohkawa *et al.*, 1984). It was reported that increased phytic acid consumption resulted in the reduced kidney stone formation and hypercaliciuria (Ohkawa *et al.*, 1984). The results from a recently conducted cohort epidemiological study has shown that dietary calcium intake was inversely associated with the risk of kidney stones (Curhan *et al.*, 1993). It was suggested that a high intake of calcium reduced the absorption of oxalate in the urinary excretion of oxalate resulting in reduced formation of calcium oxalate stones, and thus reduced the risk of kidney stone formation.

Phytate has also been found to inhibit platelet aggregation, pulmonary insufficiency, diabetes, antidote activity against lead poisoning (Thompson, 1984; Graf, 1986).

e. Role as second messengers

During the past two decades, the role and function of inositol containing compounds as second messengers in intracellular signal transduction systems has been extensively studied (Berridge and Irvine, 1989; Brass and Joseph, 1985; Challiss *et al.*, 1991; Irvine *et al.*, 1986; Kohayashi *et al.*, 1988; Michell, 1986a, 1986b; Morris *et al.*, 1987; Meyer and Stryer, 1990; Nishizuka, 1984; Nishizuka, 1988; Nosek *et al.*, 1986; Putney, 1987). It has been demonstrated that extracellular signals that activate cellular functions and proliferation through stimulation of cellular membrane receptors provoke the hydrolysis of membrane bound inositol phospholipid, phosotidylinositol-4,5-diphosphate (PIP_2), to

produce at least two second messengers: diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$) (Berridge, 1981; Michell, 1975). DAG acts by stimulating protein kinase C (PKC) (Nishizuka, 1988), which in turn phosphorylates a range of cellular proteins, some of which regulate cell functions. PKC has been found to serve as the receptor for phorbolesters, a class of tumor promoters (Castanga *et al.*, 1982; Yamanishi *et al.*, 1983), which may well explain the function of PKC cell growth and differentiation. $InsP_3$ functions to release calcium from internal stores (Berridge, 1987; Berridge and Irvine, 1984), with endoplasmic reticulum being the greatest source in the cells (Streb *et al.*, 1984, Figure 2.6).

The intracellular metabolism of inositol compounds is well explained by Berridge and Irvine (1989). Although many metabolic steps and many intermediate steps are involved in the formation of PA, the general agreement is that after the hydrolysis of PIP_2 and the subsequent formation of both DAG and $InsP_3$ by extracellular stimulation, $InsP_3$ is rapidly phosphorylated to $InsP_4$ (Michell, 1986). It has been suggested that $InsP_4$ functions as an additional intracellular messenger and regulates the entry of calcium ions into the cells from the external medium. Thus, $InsP_3$ functions as second messenger directly to regulate the release of intracellular calcium stores from endoplasmic reticulum and indirectly to mediate extracellular calcium entry through rapid production of its phosphorylated metabolite, $InsP_4$, in response to cellular stimulation (Zhou and Erdman, 1995). Although the intracellular importance of

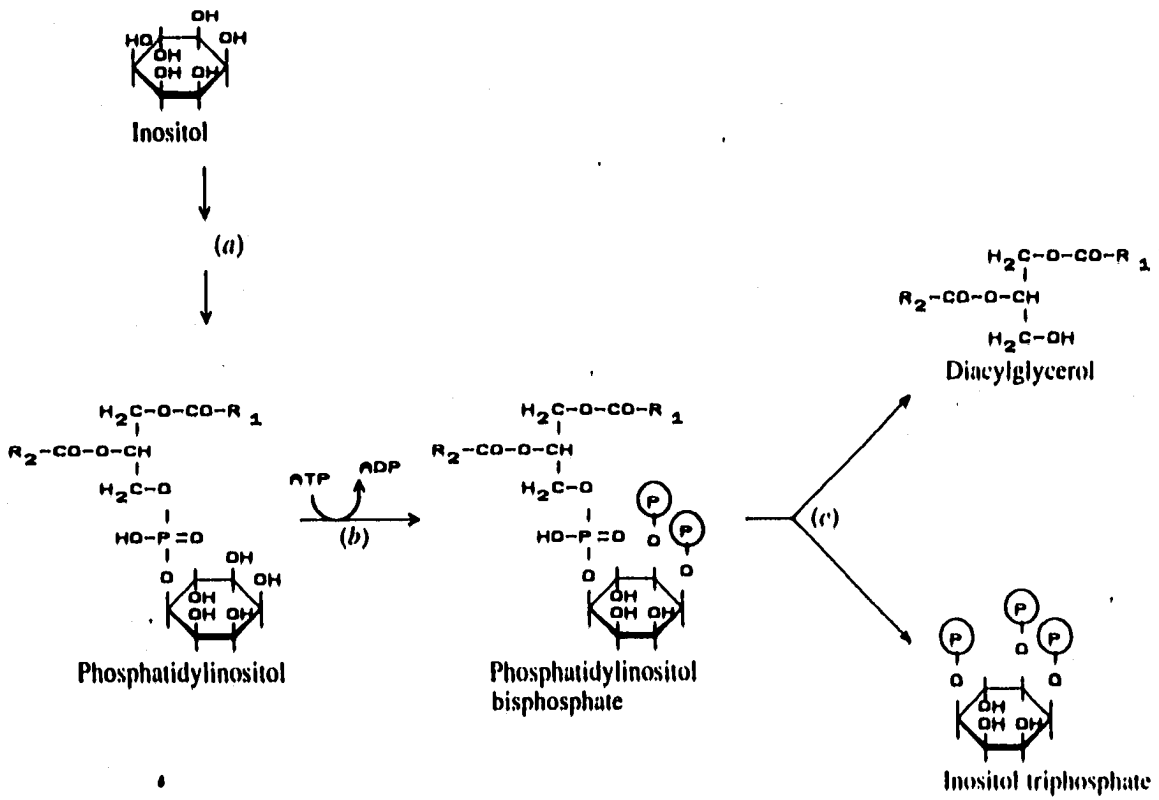

Figure 2.6 The role of phosphotidylinositol in phosphorylation . (a) reaction between inositol and diacylglycerol; (b) two successive phosphorylation reactions; (c) harmone sensitive phospholipase C. (adapted from Graf *et al.*, 1987)

higher phosphorylated $InsP_5$ and $InsP_6$ has not been clarified, they are generally considered to be agonist insensitive (Curhan *et al.*, 1993) and may act as neuromodulators extracellularly by activating neurons *in vitro* and could be novel pharmacological tools (Vallejo *et al.*, 1987).

Dietary phytic acid could serve as a precursor for second messengers *in situ* if it were absorbed and appropriately dephosphorylated. There is *in vivo* evidence that it is hydrolized in the gastrointestinal tract to lower phosphorylated forms by the action of various phytases and taken up by gastrointestinal tract mucosal cells (Berdanier, 1992).

Interest in the regulation of calcium extends beyond its second messenger functions. There exist pathological conditions such as, brain hypoxia, that can lead to an unregulated elevation of calcium (Ca^{2+}), which in turn may threaten cell viability as a result of depletion of adenosine triphosphate (ATP), the activation of Ca^{2+} mediated proteases and lipases, or other deleterious events. In such instances it may be desirable to block the elevation of Ca^{2+} in order to maintain cell viability. For these various physiological and non-physiological considerations, it is of interest to develop agents by which one may alter the intracellular phosphoinositide mediated second messenger system pharmocologically, in the hope that therapeutic agents may emerge.

f. Role in food preservation

Various studies have shown that iron-induced oxidative damage can be reduced in food by the addition of small amounts of PA (Empson *et al.*, 1991; Champagne *et al.*, 1990; Tsuno *et al.*, 1986), suggesting a food preservative

property. It also provides a significant protection against ascorbic acid degradation and slowing lipid peroxidation in chick meal (Empson *et al.*, 1991).

Although phytic acid has strong inhibitory effects on essential minerals (Ca^{++}, Zn^{++}, Fe^{++}) bioavailability in both humans and animals, it can be used for potential beneficial health effects due to its chelation properties. It can be adopted in lowering serum cholesterol and triglycerides, and preventing heart disease, renal stone formation, and certain types of cancer. The metabolites of phytic acid may also serve as second messengers and neuromodulators. Because the beneficial aspects of PA have only been derived from limited *in vitro*, animal, or epidemiological investigations, carefully controlled human and animal studies should be carried out to simultaneously evaluate its potential benefits and adverse effects.

Undoubtedly, scientific and economic interest would be necessary to find the means to decrease the rate of mutations in humans, and subsequently cancer suppression. It is interrelated to identify and introduce antimutagens or counter des-mutagenic agents through diet. Phytic acid, a common constituent of plant foodstuff especially cereals, legumes and seeds, may be hypothesised as one of those unknown components which has to be examined for their anti-mutagenicity against aflatoxins.

H. Evaluation of Mutagenicity

An upsurge of activities in the mutagenesis area has been brought about by the availability of short-term tests, partically with the introduction of Ames *Salmonella* tester strains. This assay has provided opportunities unparalleled in

the history of genetic toxicology and has been extremely helpful in recognizing the carcinogenic potential of several intrinsic components in foods and environmental chemicals. Since it is diffult to remove all of the health related hazards from the environment and/or food, the attention has been shifted to an approach based on the inhibition of mutagenesis by antimutagenic factors. The possibilty to test the antimutagenicity of a variety of compounds from plants has become easier and less time consuming by the invention of *in vitro* tests including enzyme-linked immuno assays, *in vivo* cell lines, especially by the use of *in vitro* bacterial assys such as, Ames *Salmonella* mutagenicity testing. This review discusses the use of the Ames *Salmonella* microsomal mutagenicity assay in relation to food toxicolgy.

1. *Salmonella*/Microsomal Mutagenicity Assay (Ames Test)

The Ames test is a relatively easy-to-perform method because of its availability and reliability to detect known animal carcinogens and bacterial mutagens. It has become a primary assay not only in the chemical and drug industry, but also for the safety evaluation of different treatments applied to foods and feed during the last two decades. However, it is very important to take into account that the use of mutagenicity tests to predict carcinogenesis is not perfect. Maron and Ames (1983), while making a review of this method indicated that correlation between carcinogenicity and mutagenicity was 83%. Some other studies have shown this correlation ranging between 60-95 %. Some of the known carcinogenic compounds such as polychlorinated pesticides have

been tested negative in the Ames test (Ames and McCann, 1981), but were found to be carcinogenic in some aquatic toxicity assays and other mammalian animals.

In principle, the Ames test consists of exposing one or more *Salmonella typhimurium* mutants (converted to histidine dependent) strains to the potential mutagen or sample. Either with or without metabolic activation, the tested sample or compound will revert the bacteria from histidine-dependent to histidine-independent, which will be able to grow on a histidine deficient media and counted after 48 hours of incubation at 37°C.

a. Tester strains

A set of histidine-requiring strains is used for mutagenicity testing. Each tester strain contains a different mutation in the operon coding for histidine biosynthesis. Tester strains TA97, TA98, TA100, and TA 102 contain a mutation (*rfa* mutation) that makes the lipopolysaccharide barrier coating the surface of the bateria more permeable to larger molecules, therefore enhancing the penetrability of potential mutagens (Ames *et al.*, 1973). Another mutation present in these tester strains (except TA102) is the *uvrB* mutation. This mutation is a deletion of the gene coding for the excision repair system, which causes a permanency of the effect of the mutagens; therefore, it increases the sensitivity of the strains (Ames *et al*, 1973). This mutation is found in the gene coding for biotin synthesis; as a consequence, these bacteria also require biotin for their growth. In order to increase their sensitivity, these tester strains contain

an R-factor plasmid pKM101, which enhances an error-prone DNA repair system and increases the chemical and spontaneous reversion rate of the strains (McCann *et al.*, 1975; Levin *et al.*, 1982).

The histidine mutation in TA 100 is found in the sequence coding for the first enzyme in the histidine biosynthesis pathway. This mutation substitutes GC, GC, GC (histidine independent) for GC, AT, GC (histidine dependent) (Barnes et al. 1982). As a consequence, TA100 tester strain detects mutagens that cause a base-pair substitution type of mutation, which restore the right sequence for production of histidine. The histidine mutation in TA98, which is found in a sequence coding for histidinol dehydrogenase, consists of a shifted pairing that occurs in repetitive sequence or "hot spots" (in this case, repetitive GC). This strain detects various mutagens that cause frameshift type of mutation that restore the right sequence for histidine biosynthesis (Isono and Yourno, 1974).

Maron and Ames (1983) recommend confirming tester strain genotypes when bacteria have been received, when a new frozen or lyophilized permanent has been opened, right before performing a mutagenicity test, when spontaneous revertants fall out of normal range, or when the sensitivity to standard mutagens has become weakened or lost. For this purpose, a set of biochemical tests is performed for the confirmation of histidine requirement by growing bacteria in selective agar. It includes: the testing of UV light sensitivity (*uvrB* mutation), sensitivity to grow in a crystal violet containing agar (*rfa* mutation), and testing the resistance to antibiotics (ampicillin for TA98 and TA100, and tetracycline

for TA102) present in media (R-factor). All tester strains were originally derived from *Salmonella typhimiurium* LT2. Although TA98 and TA100 are the most commonly used tester strains to evaluate the aflatoxin-induced muatagenicity (Droughton and Childs, 1982; Hrelia *et al.*, 1996; Jorgensen *et al.*, 1990; Balanski, 1992; Rojanapo and Tepsuwan, 1993; Weng *et al.*, 1997), other *Salmonella* tester strains TA98, TA100, and TA102 are recommended for general mutagenesis testing (Maron and Ames, 1983).

b. Spontaneous reversion

The number of spontaneous (natural) revertants from histidine dependent to histidine independent should be obtained routinely in every mutagenesis experiment and expressed as the number of natural or spontaneous revertants per plate. The rate of spontaneous reversion is characteristic for each tester strain. The number of spontaneous revertants after 48 h of incubation depends on the histidine concentration. However, spontaneous reversion is independent of the initial number of cells plated, within a certain range (10^5 to 10^8 cells). Even though the number of natural revertants obtained from one experiment may be different from that obtained in others, or even within the same experiment, it is recommended to run at least three spontaneous revertant control plates in every experiment from each strain (Maron and Ames, 1983). The normal ranges for spontaneous revertants without metabolic activation cocktail (S9 mix) are: for TA98, 30-50 revertants/plate; TA100 (120-200 revertants/plate; TA102, 240-320 revertants/plate. Abnormally high spontaneous revertant numbers may

indicate contamination or accumulation of back mutation by repeating subculturing. A decrease may indicate partial or complete loss of the R-factor (if they are sensitive to ampicillin). Another factor, which may cause variability in the number of spontaneous revertants, is the nutrient broth. Difco nutrient broth has been found to cause mutations in tester strain TA100 without metabolic activation. In general, nutrient broth that contains beef protein extracted at high temperatures may be mutagenic (Maron *et al.*, 1981; Maron and Ames, 1983).

c. Enzymatic metabolic activation

Many compounds require biotransformation to reactive metabolites in order to require mutagenic activity i.e., aflatoxins, which are called indirect-acting mutagens. In this assay, both cytosolic and microsomal enzymatic fractions are used to transform a compound into a reactive metabolite. In general, microsomal enzymes fraction (S-9) is used for the purpose of metabolic activation.

d. Preparation of S-9 microsomal fraction

The S-9 mix is an enzymatic suspension that is obtained by centrifuging induced-liver homogenates at 9000 xg and saving the supernatant (S-9) by decanting it. This suspension contains several microsomal enzymes, and the mixed function oxidase system (cytochrome P450) among them are responsible for the transformation of the parent compound in the reactive metabolite. For general mutagenesis experiments, it is recommended to use liver homogenates

from rats that have been previously induced with a polychlorinated biphenyl (PCB) mixture, such as Aroclor 1254 (Alvares, *et al.*, 1973; Schmoldt *et al*, 1974). The role of Aroclor 1254, as a with any other inducer chemical, is to promote the production of metabolic enzymes in the exposed liver in order to achieve a higher protein (enzymes) concentration when preparing the S-9 mix (Alvares *et al*, 1973). The purpose of the inclusion of a metabolic activating mixture in the mutagenesis assay, is to try to mimic the metabolic processes that the testing compound or sample would go through when it reaches the mammalian liver. Differences in the metabolic activity of S-9 preparations have been found to depend on the liver source and the inducer used (Ames *et al.*, 1973).

e. Types of assays

The plate incorporation procedure is the most commonly used type of assay (Droughton and Childs, 1982; Jorgensen *et al.*, 1990; Balanski, 1992; Rauscher *et al.*, 1998; Rojanapo and Tepsuwan, 1993 Weng *et al.*, 1977). This procedure consists of combining the sample or testing compound with the tester strain in the molten top agar, then the S-9 mix (if required) is added, and then this mixture is poured onto a minimal sucrose agar plate. After 48 hours of incubation at 37°C the revertant colonies are counted. However, some mutagens are poorly detected in the standard plate incorporation assay; they require a more sensitive method. The most widely used procedure first described by Yahagi *et al.* (1975) which includes a pre-incubation of the mutagen or sample, the S-9

mix, and bacteria at 37°C for 20-30 min and then combined with the top agar to be poured onto the minimal glucose agar plate. The increase in sensitivity has been attributed to the fact that the test compound or sample, S-9 and bacteria, are incubated at higher concentrations than those occurring in the standard plate incorporation (Prival *et al.*, 1979).

f. Diagnostic mutagens

For each mutagenicity test, a positive control must be included. This control helps to confirm the reversion properties of the tester strains and the efficacy of the metabolic mixture. There are some of the positive controls routinely used that do not require metabolic activation, i.e., N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and sodium azide (NaN_3) for TA100 and daunomycin for TA98. Positive controls that require metabolic activation are: 2-AF, and B[a]P for both tester strains.

g. Carrier solvents

Compounds tested for mutagenicity are commonly dissolved in dimethyl sufoxide (DMSO) when they are not soluble in water. In general, DMSO is the most useful carrier solvent because of its capability of dissolving a wide range of chemicals, it is relatively non-toxic to the bacteria, it does not affect the microsomal enzymes, and it is miscible in the molten top agar. However, there are compounds that may not be soluble or stable in DMSO (Szmant, 1971). Therefore, the potential of other solvents for being used as carrier solvents in

this assay has also been studied (Maron *et al.*, 1981). Thirteen additional solvents have been evaluated for their compatibility with this test system, and 12 were found to be satisfactory under certain restrictions: glycerol formal, dimethyl formamide, formamide, acetonitrile, ethanol (95%), acetone, ethylene glycol, dimethyl ether, 1-methyl-2-pyrrolidinone, p-dioxane, tetrahydrofurfuryl alcohol and tetrahydrofuran.

3. EVALUATION OF THE ANTI-MUTAGENIC POTENTIAL OF PHYTIC ACID, LINOLEIC ACID AND PHOSPHOTIDYL-INOSITOL AGAINST DIRECT-ACTING AND INDIRECT-ACTING MUTAGENS IN AMES *SALMONELLA*/MICROSOMAL MUTAGENICITY ASSAY

A. Introduction

Cancer has remained one of the leading causes of death in humans worldwide claiming more than six million lives every year whereas diet and lifestyle related cancers account for 40% of the total cancer incidences (Pezzuto, 1997). Dietary mutagens include naturally-occurring toxicants, alkaloids from plants, heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs) and generated by-products, i.e., *N*-nitroso compounds, in addition to cigarette smoking, alcohol, industrial chemicals and natural epidemics. The search for antimutagenic agents is very important since mutagenic and carcinogenic factors are omnipresent in the human environment and elimination of all of them seems to be impossible. Moreover, several well-known mutagenic risk factors are closely connected with a modern lifestyle and their entire eradication may be very burdensome and even unattainable.

Antimutagenic agents are natural or synthetic compounds which are able to lower or eliminate genotoxic effects of mutagenic or carcinogenic factors through diverse modes of action. The best candidate appears to be natural diet-components, taken in sufficient antimutagenic concentrations during regular daily meals. Extensive research in natural plant substances has identified innumerable natural antimutagenic components. It is well known, for instance,

that fruits and vegetables contain a marked amount of anticarcinogenic and antimutagenic components. The list of the most effective natural antimutagens includes: polyphenolic compounds, vitamins, minerals, flavonoids, catachin, soybean proteins, carotenoids, thiocyanate, glutathione, diallyl sulfides and others. These factors counteract the mutagenic effect by either reacting directly with mutagens (desmutagens) or by suppressing cellular mutagenesis (bio-antimutagens). Currently, the role of plant-derived dietary fiber in the protection of human mutagenesis and carcinogenesis has been extensively studied. The major sources of dietary fiber are whole cereal grains or bran-milling fractions. Recently, phytic acid (*inositol hexaphosphate*) and other components of phosphorylation other than fiber have been identified and tested for their anticancer properties in animal studies. It has been assumed that phytic acid, which is an instrinsic component of grains, can render protection against cancer through its antioxidant properties (Graf, 1985; Shamsuddin, 1995).

Phytic acid is a naturally occurring compound in cereals and legumes (0.4-6.4%). It primarily exists as a salt with mono- and di-valent cations (Ca^{++}, Na^{+}, Mg^{++}, K^{+}) and is considered the chief storage form of phosphorous for germinating seeds. Not surprisingly, it has been considered an anti-nutritive factor due to its chelating property with important minerals (Cu > Zn > Mn > >Fe > Ca, in decreasing order), and therefore inhibits the mineral absorption. Paradoxically, the ability of phytate to bind with metals especially iron (Fe^{2+}), may be responsible for phytic acid as an antioxidant resulting in anticarcinogenic activity. By chelating Fe^{2+}, phytic acid inhibits Fe^{2+}-induced

free radical (.OH) generation (Graf and Eaton, 1990). This is achieved by occupying all the available Fe^{2+}-coordination sites, thus inhibiting .OH generation from the Fenton reaction. Subsequently, it limits the processes of lipid peroxidation and DNA damage by inhibiting free radicals formation, which are thought to be involved in the etiology of certain cancers (Shamsuddin, 1995).

Research on the role of inositol phospholipid derived molecules in plants has focused on the presence of phosphotidylinositol (PI) and its phosphorylated products, i. e., phosphotidyl inositol 4, phosphate (PIP_2), inositol-4,5,6-phosphate ($InsP_3$) and diacylglycerol (DAG) in plant cells (Verhey and Lomax, 1993; Chattaway *et al.*, 1992). The characterization of enzymes for inositol phospholipid metabolism and the effects of DAG and $InsP_3$ in plant cells has focused on the role played by these compounds in plant growth and cell-to-cell communication. Inositol hexaphosphate (phytic acid) and other higher phosphates are the phosphorylated products of the same PI bio-transformation pathway. PI is two times (8-15% molecule) more abundant in plant membranes than animal membranes (Verhey and Lomax, 1993). In contrast, phosphorylated forms of PIP_2, PIP_1, and PIP are present in lower amounts while enzymes, i.e., PIP-hydroxy kinase which cleaves PIP_2 to $InsP_3$ are also found in plants cells. Phospholipase C (PLC), an enzyme which is specific for inositol phospholipids, cleaves the phosphodiester bond of PIP_2 and releases $InsP_3$ and DAG. $InsP_3$ is further dephosphorylated to higher InsP or takes part in signal transduction whereas, DAG activates protein kinase C (PKC) (Mistry *et al.*, 1995). Polyunsaturated fatty acids, i.e., linoleic, linolenic, palmitic acids are connected

with inositol through a phosphodiester bond (Chattaway *et al.*, 1992). Furthermore, it has been shown that these polyunsaturated fatty acids play an important role in activating and/or inactivating the cytochrome P-450 (CP-450) enzyme system required for the biosynthesis of certain chemicals to become mutagenic (Graf and Eaton, 1990; Mistry *et al.*, 1995).

Since phytic acid, PI and linoleic acid are found at various stages of plant growth and are present in substantial amounts, their role in chemoprevention cannot be underestimated. The objective of this study was to explore the potential correlation between phytic acid, linoleic acid, and phosphotidylinositol individually or in combination against the mutagenic activity of direct-acting, Methyl nitro-*N*- nitrosoguanidine (MNNG), and sodium azide (NaN_3) and indirect-acting, aflatoxin B_1 (AFB_1) and 2-aminoflouorene (2-AF), mutagens. In addition, in the process of conducting these studies, different salts of phytic acid were also tested against these mutagens.

B. Materials and Methods

1. Chemicals

D-biotin, L-histidine, ampicillin, tetracycline, sodium dihydrogen phosphate, β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), and glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Citric acid monohydrate, potassium phosphate, dibasic, anhydrous, potassium chloride, chloroform, methylene chloride, methanol, dimethyl sulfoxide, and water (HPLC grade) were purchased from EM Science Inc.

(Cherry Hill, NJ). Bacto agar and crystal violet were obtained from Difco-Laboratories (Detroit, MI). Glucose was purchased from Fisher Scientific (Fair Lawn, NJ). Acetonitrile was purchased from Baxter Scientific (Muskegon, MI). Acetone was obtained from Chempure Co. (Houston, TX). Phytic acid (Ca^{+2}, K^{+}, Mg^{+2}, and Na^{-}-salts), phoshotidylinositol, and linoleic acid were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO). Post mitocondrial supernatant (S-9 mix) was obtained from the Molecular Toxicology Inc. (Boone, NC). Methyl nitro-*N*- nitrosoguanidine (MNNG), 2-aminoflouorene (2-AF), and sodium azide (NaN_3) were purchased from Sigma-Aldrich (St. Louis, MO). Aflatoxin standards were kindly provided by Dr. Mary W. Trucksees (Food and Drug Administration, Washington, D.C.).

2. Bacterial Strains

The bacterial strains (TA-98 and TA-100) of *Salmonella typhimurium* were kindly provided by Dr. Bruce N. Ames (Department of Environmental Toxicology, University of California, Berkley, CA)

3. Anti-mutagenicity Determination

The determination of anti-mutagenicity activity towards AFB_1, 2-AF, MNNG, and NaN_3 was carried out by using the *Salmonella* microsomal mutagenicity assay (Ames test) using tester strains TA-100 and TA-98 with and without metabolic activation (S-9) as described by Maron and Ames (1983). Phytic acid (various salts), phosphotidylinositol (product of inositol and linoleic acid) and linoleic acid at various concentrations were tested for their antimutagenic properties against AB_1, 2-AF, MNNG, and NaN_3 using the plate

incorporation procedure of the Ames test. All the assays were carried out in triplicate.

a. Preparation of standard mutagens

2-AF, AFB_1 and MNNG were dissolved in small volumes of DMSO while NaN_3 was dissolved in water. Serial dilutions of all mutagens were made to assay the standard curves at different concentrations for each compound.

b. Preparation of treatments

Different concentrations (1, 10, 100, 1000 ug / plate) of various phytic acid salts were prepared in HPLC grade sterile water. Linoleic acid and phosphotidylinositol with different concentrations were dissolved in DMSO. These solutions were then assayed using the plate incorporation procedure of the *Salmonella* microsomal mutagenicity assay against all mutagens in triplicate.

c. Biochemical tests and plate-incorporation method

The samples were tested by using *Salmonella typhimiurium* tester strains TA-100 and TA-98, according to the standard plate incorporation method described by Maron and Ames (1983). Frozen cultures were plated on ampicillin master plates and incubated for 48 h at 37°C. A single colony was then inoculated into 50 ml sterile Oxoid Broth No. 2 and incubated in the dark at 37°C for 10-14 h using a (200-250 x g) shaker water bath. After incubation, growth was confirmed by checking the turbidity using a spectrophotometer (Spectronic 20D, Spectronic Instruments, Rochester, NY) at 650 nm using Oxoid Broth No. 2 as a blank. Absorbance reading ca. 0.8 indicated an optimal

cell density of 1-2 x10^9cells/ml. The tester strains were tested to confirm the presence of the genetic markers using the following assays:

- Histidine requirement: a positive result of this test (grown in histidine/biotin plates); no growth in non-histidine/biotin plates showed that bacteria had not been altered as a result of handling or storage conditions and that the mutant form was present.
- Crystal violet (rfa): The results of this test showed a zone of inhibition around a disk impregnated with crystal violet. The positive test results indicated that the permeability of the membrane was present.
- Ampicillin resistance (R-factor): Tester strains grew on ampicillin plates indicating that the genetic markers for ampicillin were present.

After the bacteria tested positive for all the genetic markers, the plate incorporation assay was performed as outlined by Maron and Ames (1983). The experimental protocol is depicted in Figure 3.1. Briefly, 2 ml top agar were placed in sterile culture tubes and kept at approximately 40° C in a water bath. 100µl of treatment samples and 100 µl of standard carcinogen/mutagen were placed in each tube, 100 µl of bacteria (tester strain TA-100 or TA-98) and 500 µl S-9 mix were added (only in case of indirect acting AB_1 and 2-AF controls). The tubes were mixed and poured on minimal glucose agar plates. All samples were tested in triplicate. Water and DMSO were used as negative controls. Pure AFB_1 (1, 10, 25, 50,100, 250 ng/plate), 2-AF (1, 5, 10, 50, and 100 µg/plate), MNNG (0.1, 1, 3, 5, and 10 µg/plate) and NaN_3 (0.1, 1, 3, and 5 µg/plate) were

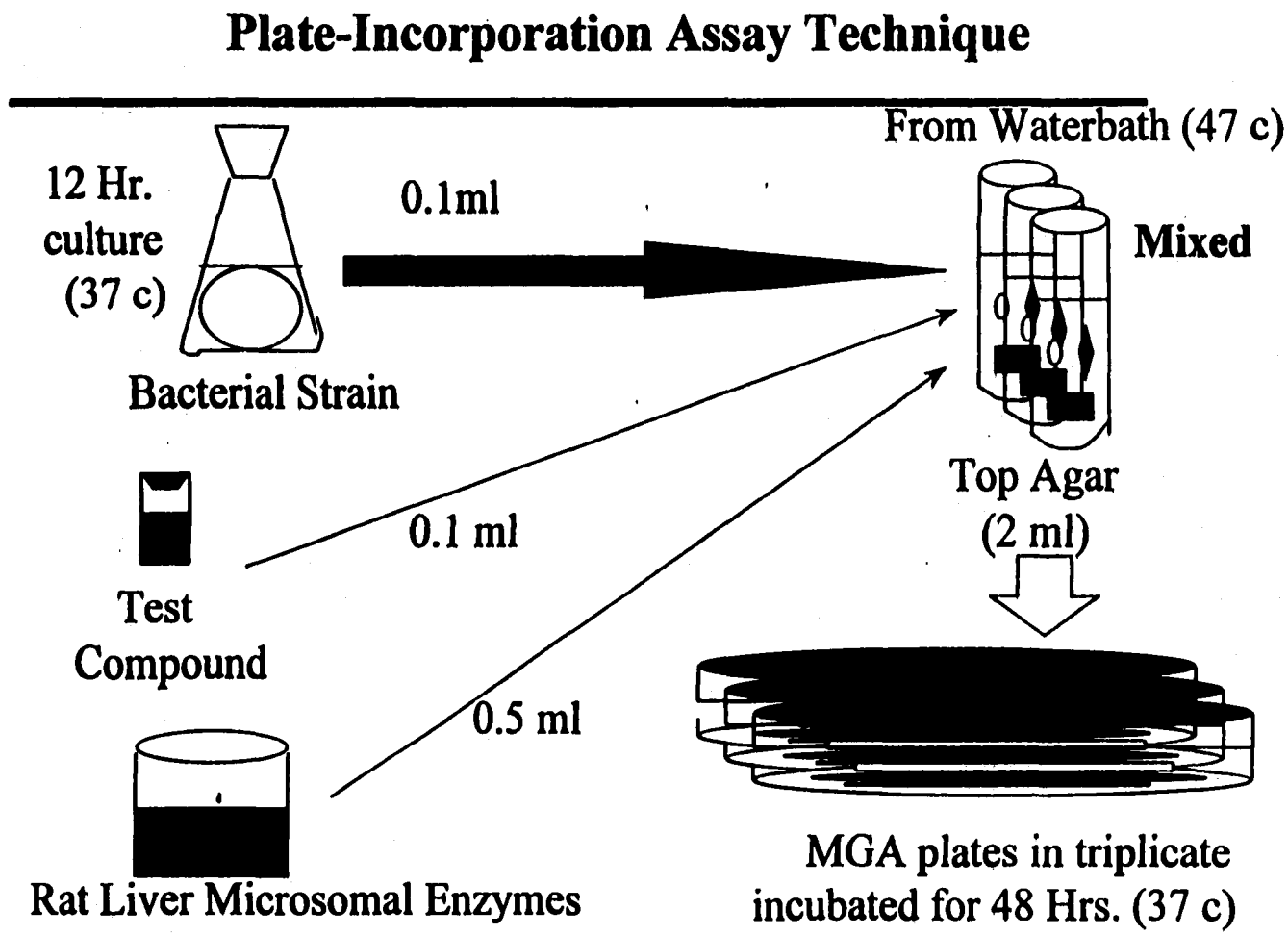

Figure 3.1 Ames *Salmonella*/microsomal mutagenicity assay using the plate-incorporation technique

used as positive controls. The plates were left at room temperature until the top agar solidified; then, they were inverted and incubated at 37°C for 48 h. After incubation, revertant colonies were counted. The number of revertants were visually counted using a Bactronic colony counter, model C-110, New Brunswick Scientific Co. (New Brunswick, N. J.) and the number of revertants were compared with standard curve.

The standard curves were obtained for both tester strains (where mentioned) using different AFB_1, 2-AF, MNNG, and NaN_3 concentrations (Figures 3.2 - 3.5). Samples with revertants over double the number of natural revertant colonies were considered mutagenic.

4. Safety Measures for Handling MNNG, 2-AF and NaN_3

Due to risks posed by 2-AF, MNNG and NaN_3, special safety measures recommended by the Louisiana State University (LSU) Campus Safety Office were followed. That includeds: (1) restricted access to the laboratory where these chemicals were going to be used during the time the test was conducted; (2) only safety-trained researchers were allowed to handle these chemicals; (3) workers had to wear appropriate personal protective equipment that included body suits, double nitrile-gloves, organic vapor-respirator and safety goggles; (4) the assay should be limited to one tester strain and using the minimum of samples possible in order to shorten the time of exposure; and (5) all the waste (material, chemical and biological) produced from this experiment should be properly labeled and disposed by LSU Campus Safety Office Personnel. For the

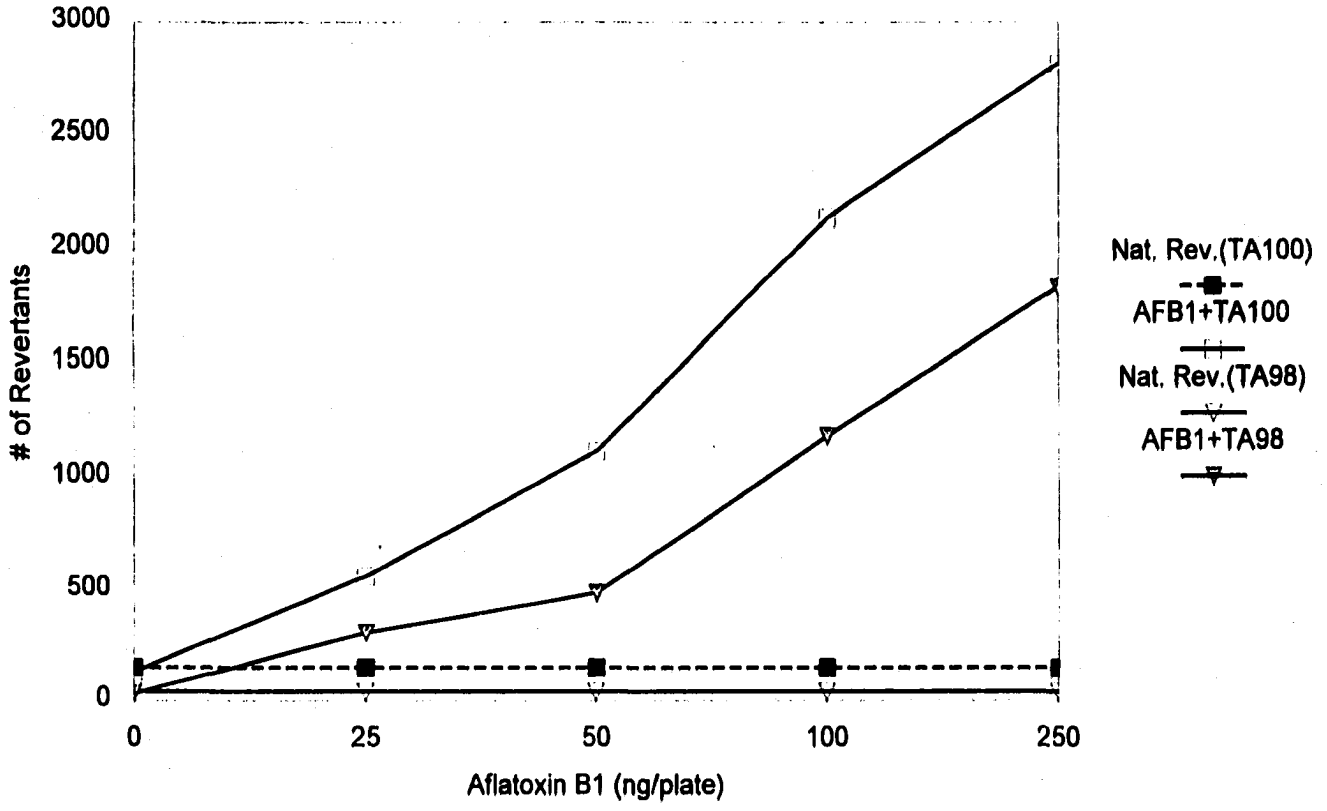

Figure 3.2 Standard curve for AFB_1 using *Salmonella* microsomal mutagenicity assay (TA98 & TA100) with metabolic activation (S-9). Values are mean +/- standard error of three replications.

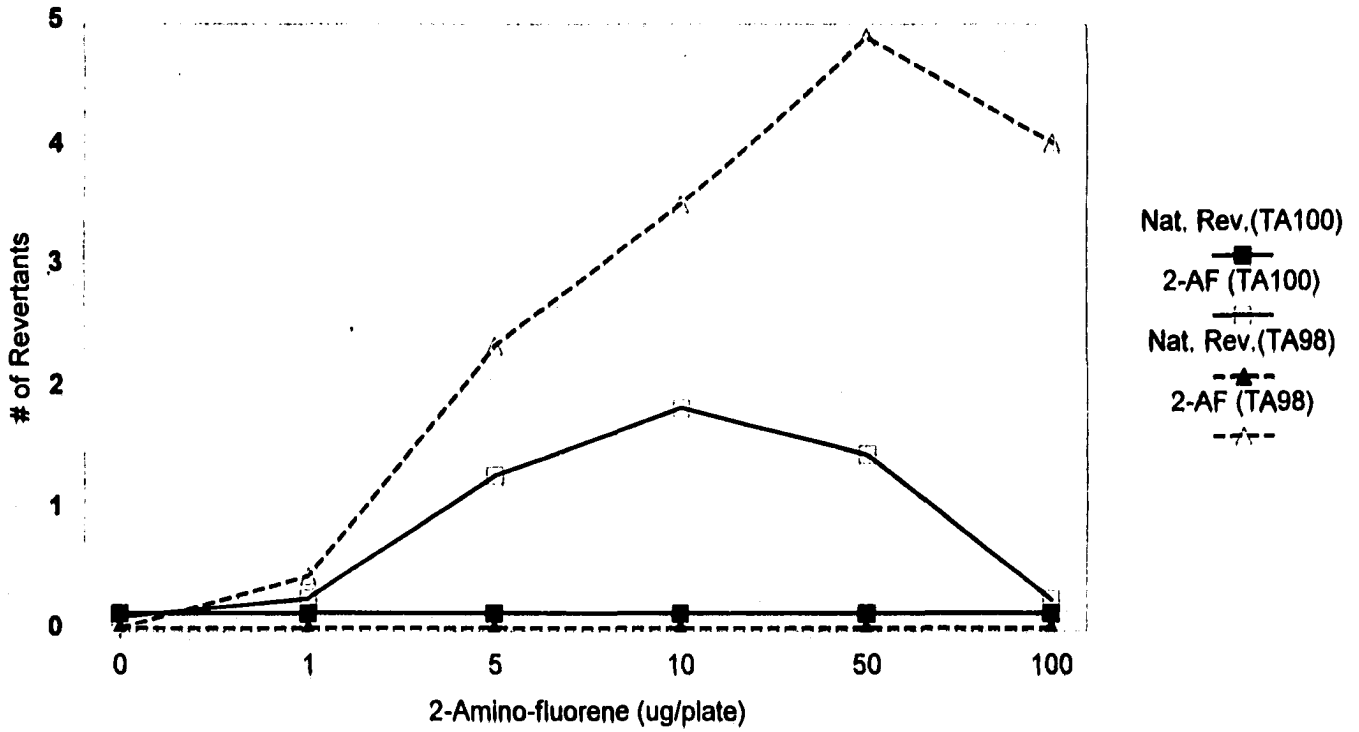

Figure 3.3 Standard curve for 2-aminofluorene in Ames *Salmonella* microsomal mutagenicity assay (TA98 and TA100) with metabolic activation (S-9). 2-AF=2-aminofluorene (5ug/plate) in DMSO. Values are mean +/- standard error of three replications.

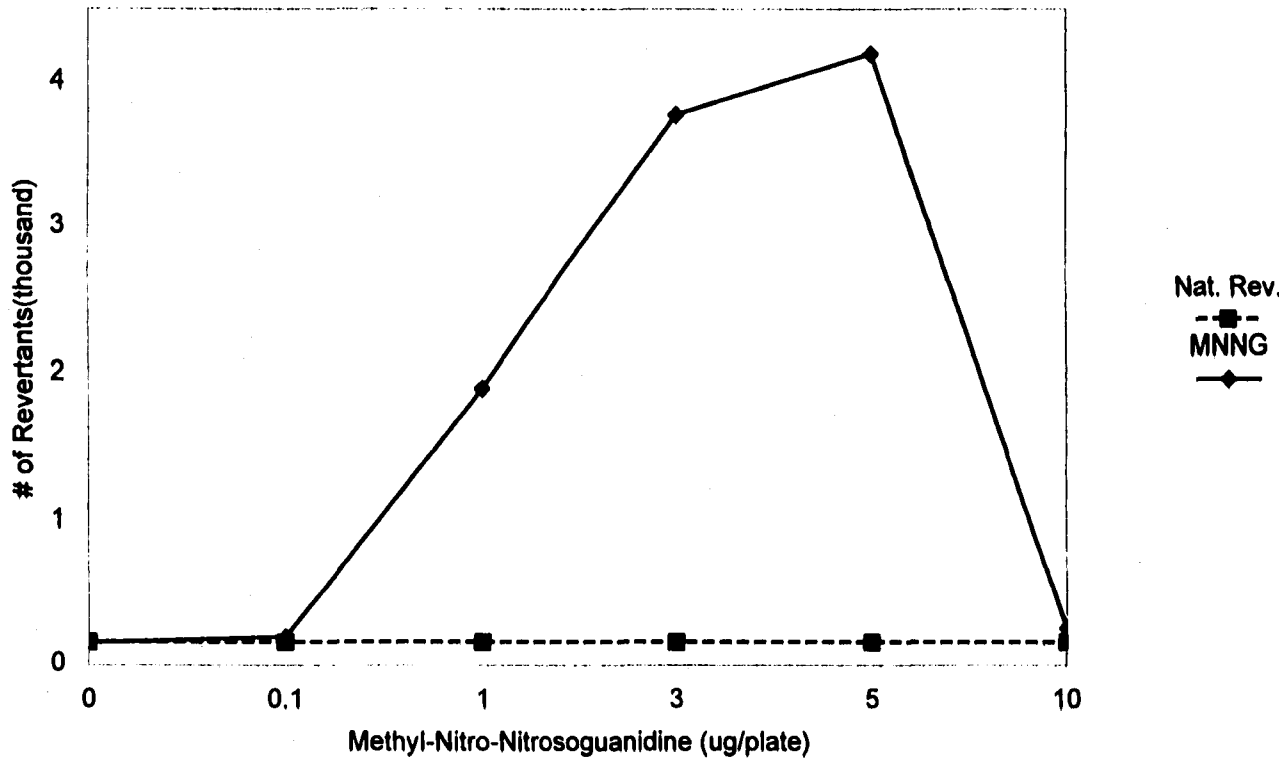

Figure 3.4 Standard curve for methyl N-nitrosoguanidine in Ames *Salmonella* microsomal mutagenicity assay (TA-100) without metabolic activation (S-9). MNNG-methyl-nitro-nitrosoguanidine (1ug/plate)in DMSO. Values are mean +/- standard error of three replications.

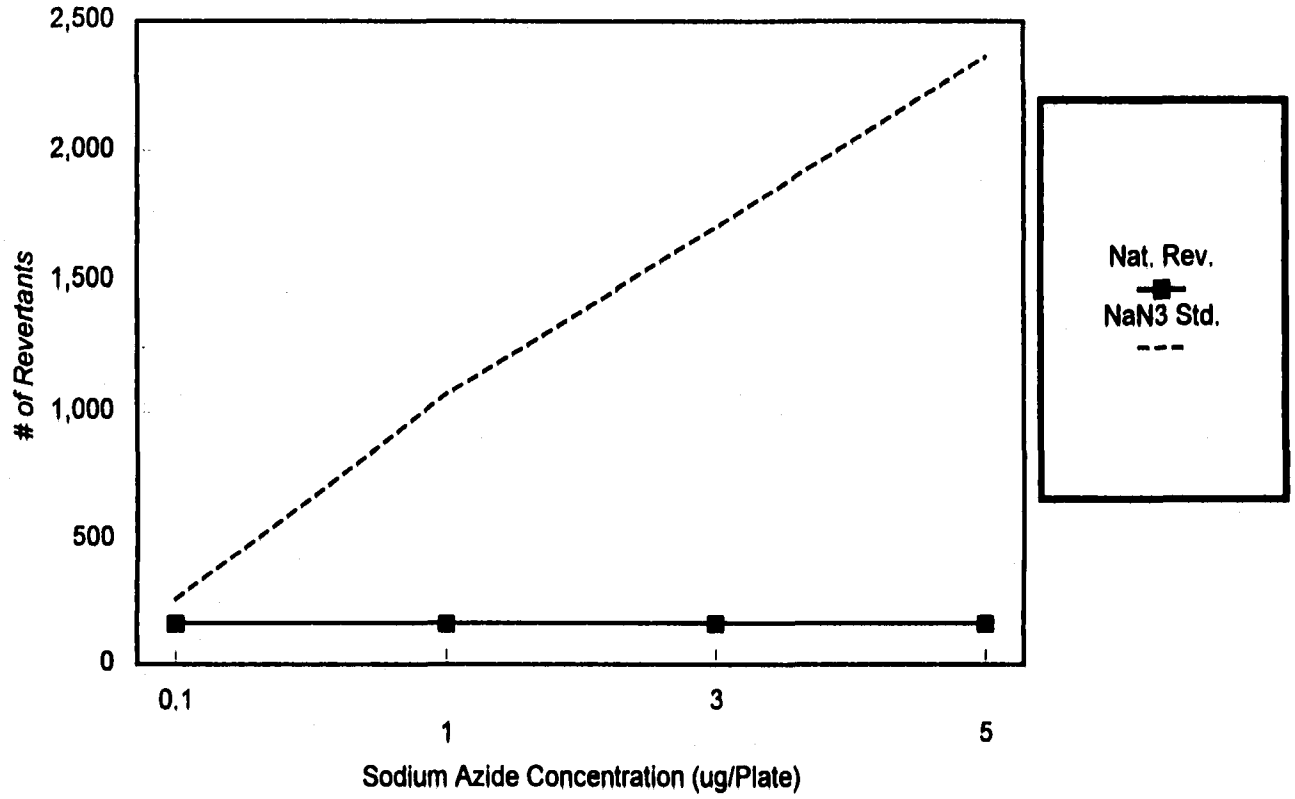

Figure 3.5 Standard curve for sodium azide (NaN_3) in Ames *Salmonella* microsomal mutagenicity assay (TA-100) without metabolic activation (S-9). Values are mean +/- standard error of three replications.

assay involving the use of these mutagens, a special protocol was designed and followed for the safety purpose.

a. Protocol for the handling of MNNG

Due to the extremely hazardous character of MNNG, a special protocol was designed and followed for its handling before, during, and after the anti-mutagenicity assay where this compound was involved. The MNNG container was opened only within a biological laminar flow hood and wearing the appropriate Personal Protective Equipment (PPE) such as body suits, double nitrile-gloves, organic vapor-respirator and safety goggles.

1. All the material and equipment used to weight the MNNG had to be placed on a water-wetted surface (*i.e.*, paper towel) and all the glassware used was disposed.

2. The MNNG was extracted from the container with a small metallic spatula and deposited inside of a flask. This flask was pre-placed on a scale previously covered with a wet filter paper. In case of any spill, MNNG (a watersoluble compound) could stick to the protective wet surfaces. After use, the spatula was wiped with a wet towel.

3. An appropriate pre-measured volume of DMSO was poured into the flask to make the required MNNG solution. After the MNNG container was closed and the solution prepared, the secondary pair of gloves was changed for a new pair.

4. From the MNNG solution (stock solution) necessary dilutions were made using disposable pipettes and glass vials. After the required diluted solution was prepared, a secondary pair of nitrile-gloves was changed again.

5. With the diluted MNNG solution, the testing samples were spiked using disposable pipettes and they were left inside the hood for the test.

6. After the samples were spiked all the material and equipment was wiped with wet towels and taken out from the hood. All the material was deposited into a biohazard waste disposal cardboard box.

7. The same PPE was worn during the anti-mutagenicity assay.

8. After the assay was completed, all vials containing the spiked sample were disposed and autoclaved. Autoclaved waste was turned over to the LSU Campus Safety Office personnel for appropriate disposal.

9. MNNG was put securely in a secondary container, appropriately labeled, and stored at -20° C for further use.

b. Safety precautions for 2-aminofluorene

2-AF is considered a mutagen and because the Material Safety Data Sheet (MSDS) information on its potential toxic effects was limited, the assay was carried out with extreme caution using appropriate personal protection equipment that included body suits, double nitrile gloves, half face negative pressure organic vapor respirator and safety glasses. Sample size and number of assays performed were kept to a minimum to limit generation. 2-AF was handled using disposable glassware which was properly disposed of at the

conclusion of the assay. Waste material generated from this experiment was autoclaved and disposed through the Louisiana State University Department of Occupational and Environmental Safety once the assay was concluded.

c. Safety precautions for sodium azide

Similar safety measures were taken for the safe handling of this direct-acting mutagen as in the case of MNNG. The compound was dissolved in distilled water to prepare a stock solution under the laminar hood aseptically. Working standards were prepared by serially diluting the stock solution.

C. Results and Discussion

The effect of phytic acid (various salts) on the mutagenic activity of several standard mutagens was tested in the Ames test with *Salmonella typhimiurium* tester strains (TA100 and TA98) recommended for a particular mutagen. Two of the tested standard mutagens were direct-acting genotoxic compounds, MNNG and NaN_3, and two of them were indirect-acting; the promutagens AFB_1 and 2-AF. In the case of promutagens or indirect-acting mutagens, the mutagen-activating metabolic enzyme fraction (S-9) was added in addition to the diagnostic compound.

1. Anti-mutagenic Potential of Phytic Acid Against Direct-acting and Indirect-acting Mutagens

In the first step, the effect of commercially available phytic acid (Na-salt) was tested against the mutagenic activity of AFB_1. Different concentrations of phytic acid were used to explore a possible correlation between the antimutagenicity of phytic acid and AFB_1 mutagenicity. According to the

results no significant antimutagenic activity was found for phytic acid (Na salt) regardless of bacterial tester strains used. The inhibition of AFB_1 mutagenicity was less than 20% (no. of revertants) and phytic acid at any concentration between 1-100 μg/plate did not reduce the mutagenicity of AFB_1 (Figures 3.6 and 3.7). However, a 50% and 25% reduction in the number of revertants was evident from the 1000 μg/plate of phytic acid in tester strains TA100 and TA98, respectively. However, it is important to mention that for a compound to be called antimutagenic, the inhibition should not be less than twice the number of natural revertants.

To compare these results with a similar structural compound without phosphate group, the antimutagenic potential of *myo*-inositol was also tested in the same assay. *Myo*-inositol is the dephosphorylated form of phytic acid and contains no phosphate group. It also is the starting substrate for phytic acid phosphorylation (see chapter 2). A similar pattern of anti-mutagenicity was observed (Figures 3.6 and 3.7). No role was evident from the results of this assay that phytate could interact with AFB_1 or inhibit its mutagenicity. The results are not conclusive to state that phytic acid was anti-mutagenic, since there was not a significant difference in the number of revertants between phytic acid (Na salt) and *myo*-inositol.

In order to find out whether the antimutagenic potential was affected by the salt-type of phytic acid, calcium, magnesium, and potassium salts of phytic acid were tested against mutagenic activity of AFB_1. The results (Figures 3.8

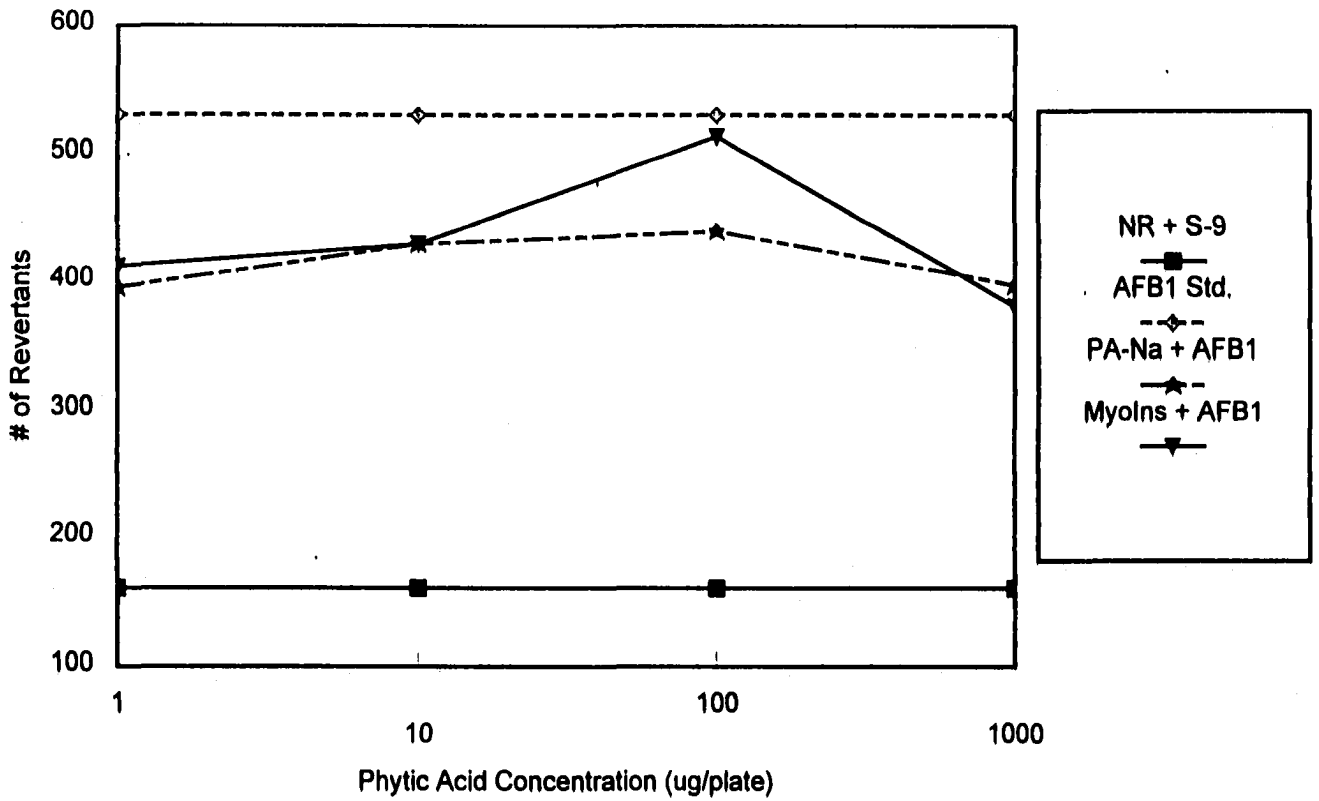

Figure 3.6 Mutagenic potential of phytic acid (Na-salt) and *myo*-inositol against AFB_1 in Ames *Salmonella* mutagenicity assay (TA-100) with metabolic activation (S-9). AFB_1=aflatoxin B_1 (25 ng/plate) in 5% DMSO. Phytic acid concentration in water. Values are mean +/- standard error of three replications.

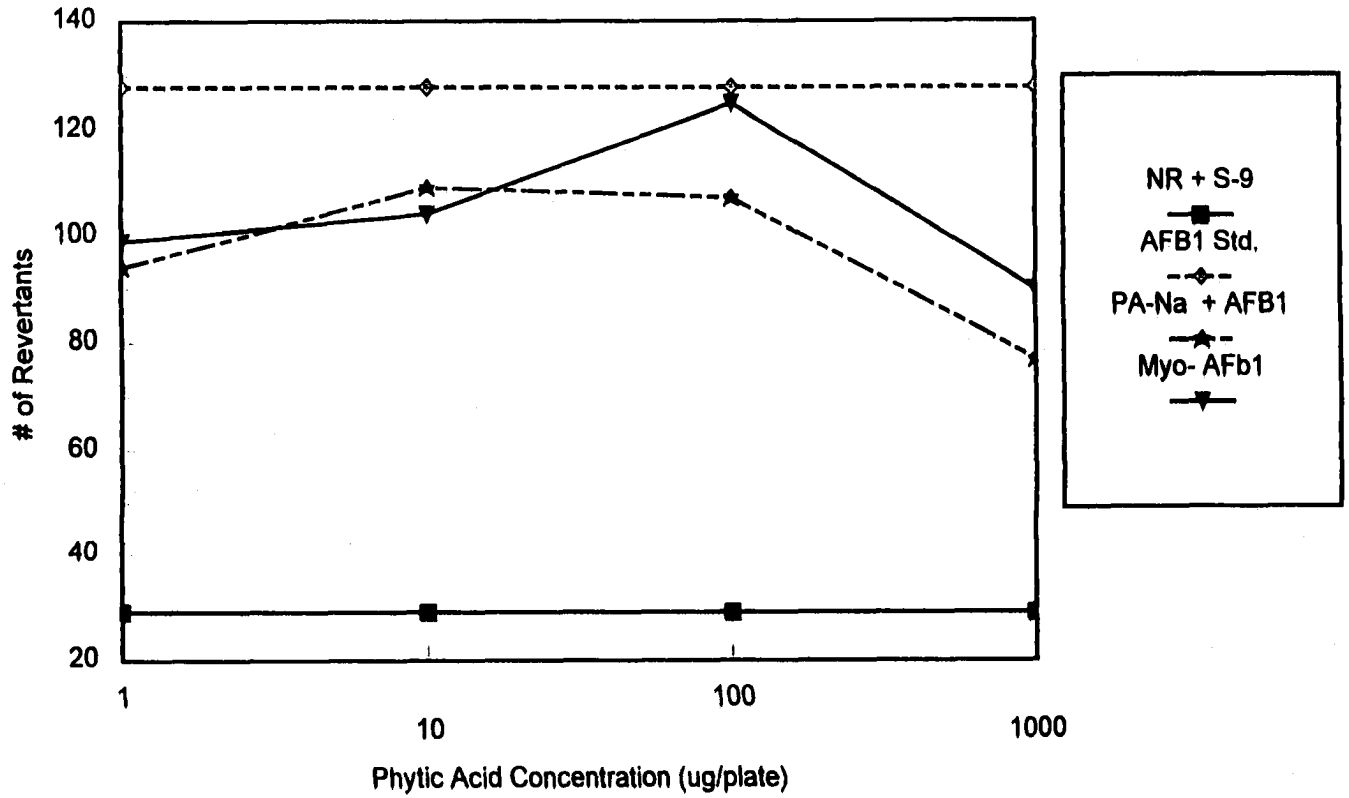

Figure 3.7 Mutagenic potential of phytic acid (Na-salt) and *myo*-inositol in *Salmonella* mutagenicity assay (TA100) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (25 ng/plate) in 5% DMSO. Phytic acid concentration in water. Values are mean +/- standard error of three replications.

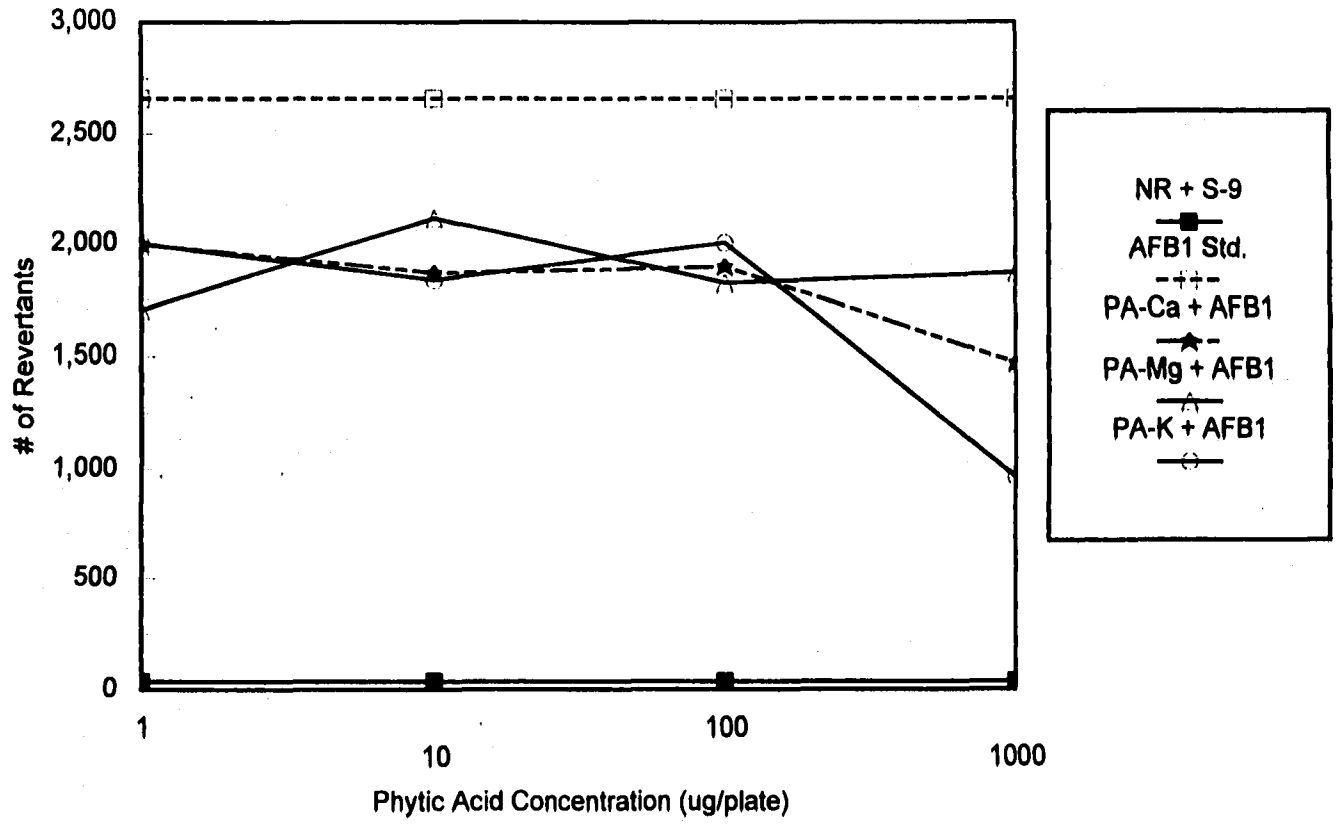

Figure3.8 Mutagenic potential of phytic acid (various salts) in *Salmonella* mutagenicity assay (TA98) with metabolic activation (S-9) against AFB_1, AFB_1=aflatoxin B_1 (100 ng/plate) in 5% DMSO. Values are mean +/- standard error of three replications.

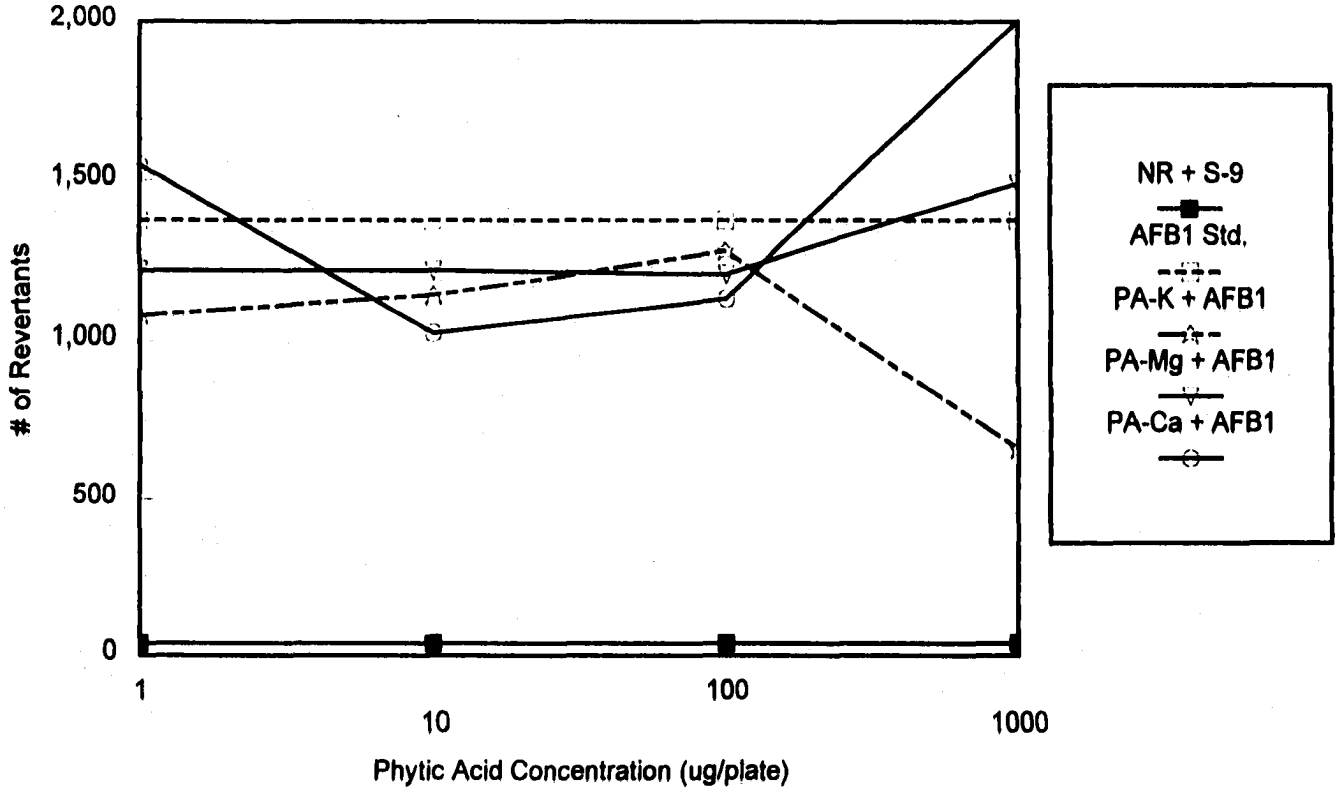

Figure 3.9 Mutagenic potential of phytic acid (various salts) in *Salmonella* mutagenicity assay (TA100) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (100 ng/plate) in 5% DMSO. Phytic acid concentration in water. Values are mean +/- standard error of three replications.

and 3.9) of this study did not show any significant difference from the previous trial. The reduction in the number of revertants were: Ca-salt (25%); Mg salt (<20%); K salt (~50%) in tester strain TA-98, whereas in tester strain TA-100, there was a non-significant reduction in the number of revertants in the case of Mg-and Ca-salts. However, K-salt resulted in >50% reduction in the number of revertants. On the basis of these results, only sodium salt of phytic acid was tested for its antimutagenic potential against 2-AF. Although the number of revertants were slightly lower (>50%) than the AFB_1 trial, the difference was not significant (Figures 3.10 and 3.11) regardless of tester strains used. These results do not show that phytic acid and its various salts are completely antimutagenic against indirect-acting AFB_1 and 2-AF. However, a 50% reduction in the number of revertants was observed in the case of the Na-, K-, and Ca-salts.

Different salts of phytic acid were also assayed against MNNG and NaN_3, which are direct-acting mutagens. In the case of the MNNG trial, Ca- and Na-salts were found to be significantly antimutagenic at 100μg and 1000μg/plate, respectively (Figure 3.12). However, Mg-salt did not show any significant reduction in the number of revertants. Although K-salt was not completely antimutagenic, the reduction in the number of revertants was significantly lower (~70%). The antimutagenic effect of phytic acid (K-salt) against MNNG, a direct-acting mutagen, suggests that the mechanism of action of phytic acid may be due to the direct interaction with MNNG.

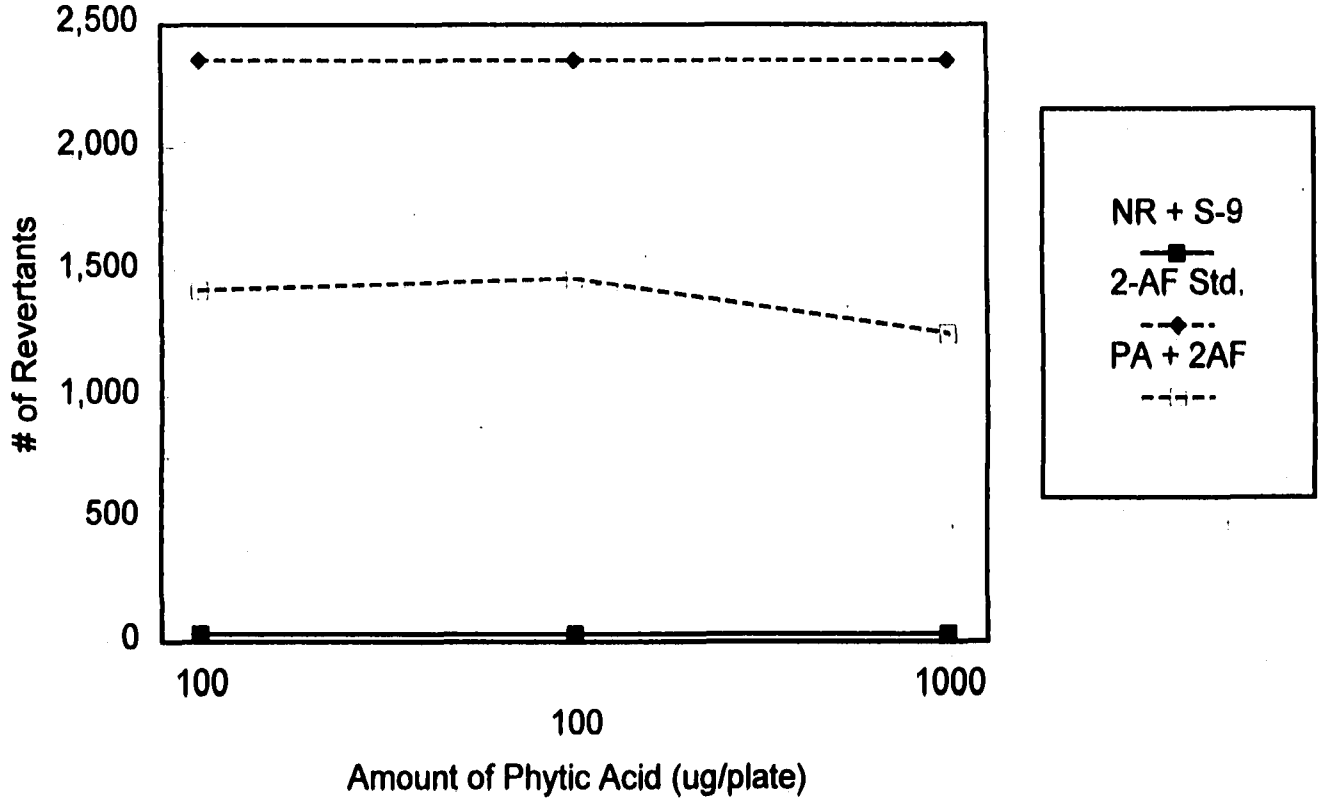

Figure 3.10 Mutagenic potential of phytic acid in *Salmonella* mutagenicity assay (TA-98) with metabolic activation (S-9) against 2-AF. 2-AF=2-aminofluorine (5 ug/plate) in DMSO. Values are mean +/- standard error of three replications.

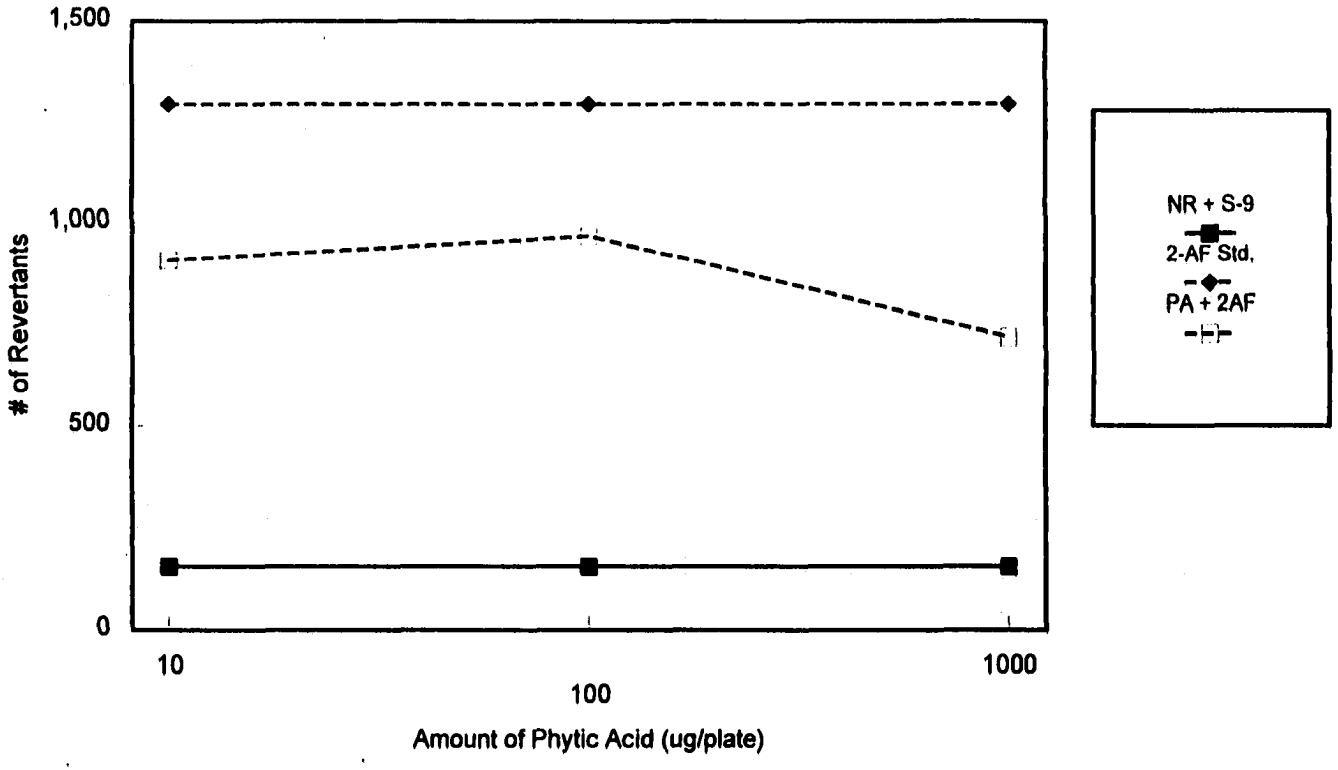

Figure 3.11 Mutagenic potential of phytic acid in *Salmonella* mutagenicity assay (TA-100) with metabolic activation (S-9) against 2-AF. 2-AF=2-aminofluorine (5 ug/plate) in DMSO. Values are mean +/- standard error of three replications.

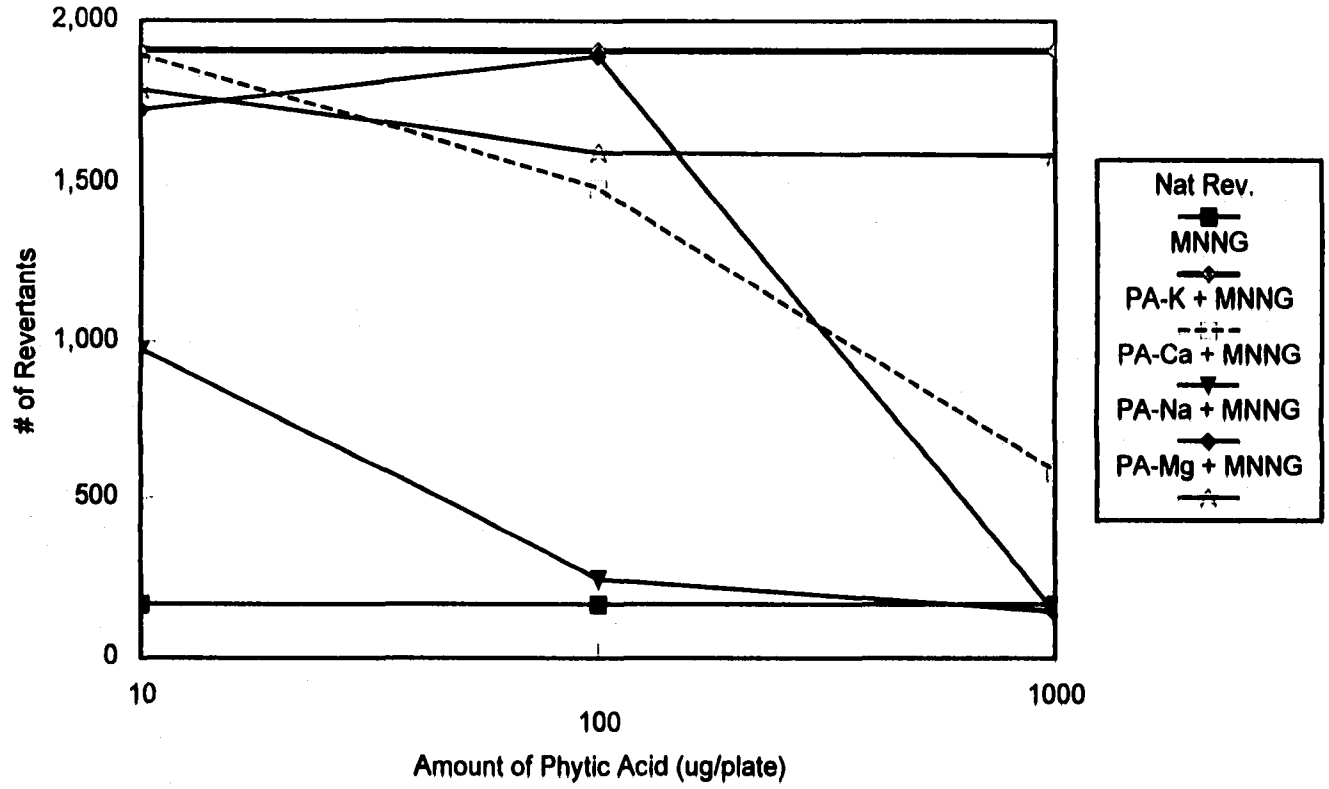

Figure 3.12 Mutagenic potential of phytic acid in *Salmonella* mutagenicity assay (TA-100) without metabolic activation (S-9) against MNNG. MNNG=methyl-nitro-nitrosoguanidine (1ug/plate) in DMSO. Values are mean +/- standard error of three replications.

In the case of the NaN_3 trial, a non-significant reduction in the number of revertants was observed for Mg-salt. However, K-, Na- and Ca-salts of phytic acid resulted in a 20-45% reduction of revertants (Figure 3.13). Ca-salt (~45% reduction in number of revertants) was the most effective of the phytic acid salts. However, these differences are not significantly different from positive control.

It is concluded on the basis of these results that phytic acid and its various salts were partially antimutagenic against indirect-acting mutagens, but were somewhat more antimutagenic against direct-acting mutagens, especially MNNG. The antimutagenic effect of phytic acid (K- and Ca-salt) against direct-acting mutagens, i.e., MNNG and NaN_3, suggests that the mechanism of action of phytic acid may be the direct interaction with MNNG and NaN_3. However, the role of different salts in exhibiting variable antimutagenic potential should be further investigated.

Previous studies have shown the effects of phytates and metal ions on the inhibition of the biosynthesis of aflatoxin in both synthetic liquid medium (Lee *et al.*, 1966; Reddy *et al.*, 1971) and in corn (Ehrlich and Ciegler, 1985; Lillehoj *et al.*, 1974), but no reports have described the potential of phytic acid to inhibit the mutagenicity of aflatoxins. Although the results of this study do not suggest that phytic acid is completely an anti-mutagenic compound in the Ames test with *Salmonella typhimirium* tester strains (TA100 and TA98), a partial reduction in the number of revertants warrants further investigations.

Phytic acid ($InsP_6$) is one of the phosphorylation by-products of phosphotidylinositol (PI) metabolism (Mistry *et al.*, 1995; Verhey and Lomax,

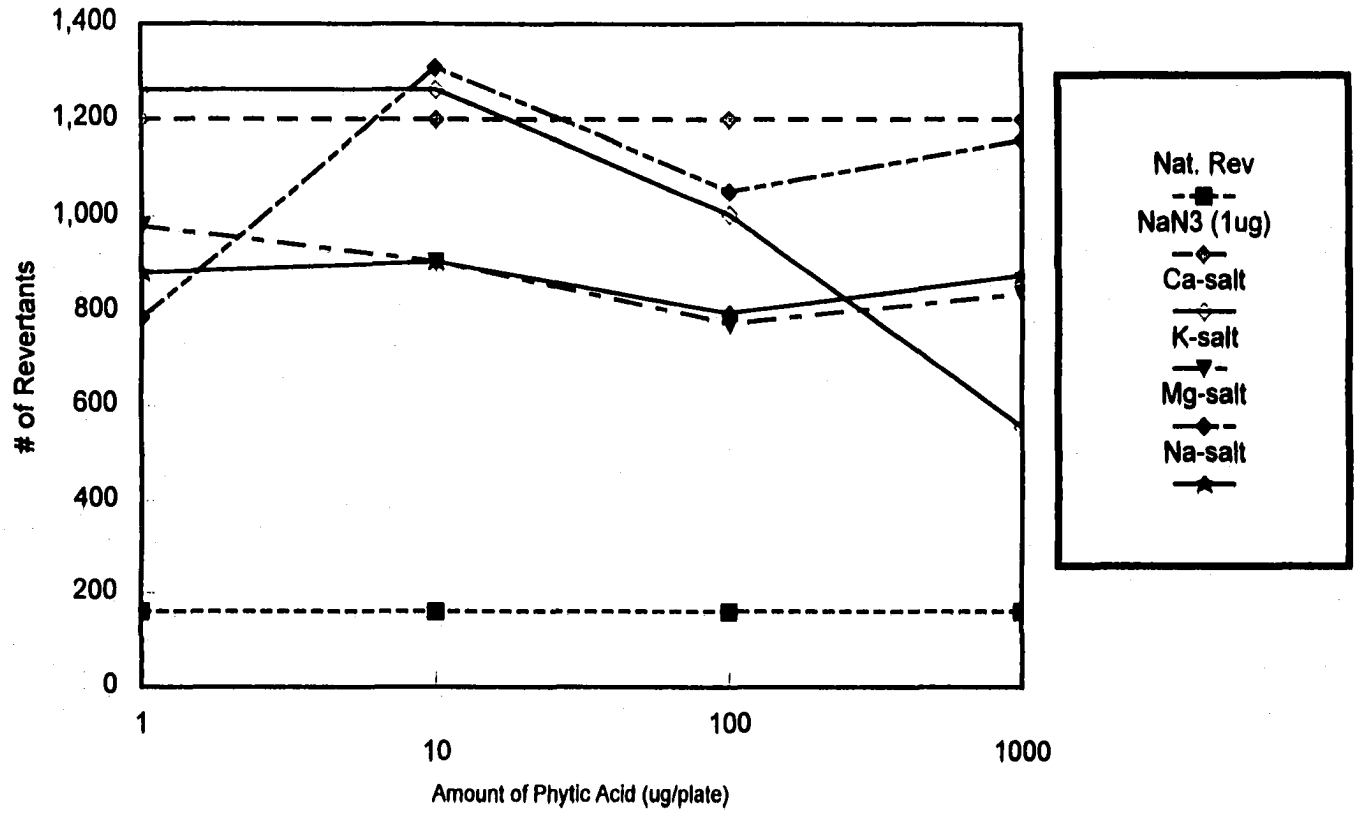

Figure 3.13 Mutagenic potential of phytic acid in *Salmonella* mutagenicity assay (TA-100) without metabolic activation (S-9) against NaN_3. NaN3=sodium azide (1ug/plate) in water. Values are mean +/- standard error of three replications.

1993). The phosphorylation of PI also results in the production of other lower and higher inositol phosphates ($InsP_2$, $InsP_3$, $InsP_4$ and $InsP_5$) (Graf and Eaton, 1990). To further investigate the antimutagenic potential of phytic acid from the point of view that its starting substrate might have the anti-mutagenic potential, the next trial was conducted by using commercially available phosphotidylinositol.

2. Anti-mutagenic Potential of Phosphotidylinositol Against Direct-acting and Indirect-acting Mutagens

Phosphotidylinositol constitutes some 5-10% of the total membrane phospholipids and is derived from the hexahydric sugar alcohol (inositol). In addition to its structural role in membranes, phosphostidylinositol has a major function in the intracellular responses to peptide hormones and neurotransmitters, yielding to intracellular second messengers, inositol triphosphate and diacylglycerol (Graf and Eaton, 1990). Their further phosphorylation results in the production of higher inositol phosphates ($InsP_4$, $InsP_5$ and $InsP_6$ commonly called phytic acid) (Graf and Eaton, 1990). PI glycans also serve as anchors for many membrane-bound proteins.

Phosphotidylinositol was assayed against the mutagenic activity of several compounds in order to confirm their potential beneficial role in the paradigm of the mutagenic process. According to the current study, PI was found to be significantly anti-mutagenic against indirect-acting AFB_1 in both tester strains of *Salmonella typhimiurium* (Figures 3.14 and 3.15). It is evident that as the concentration of phosphotidylinositol increases, the number of

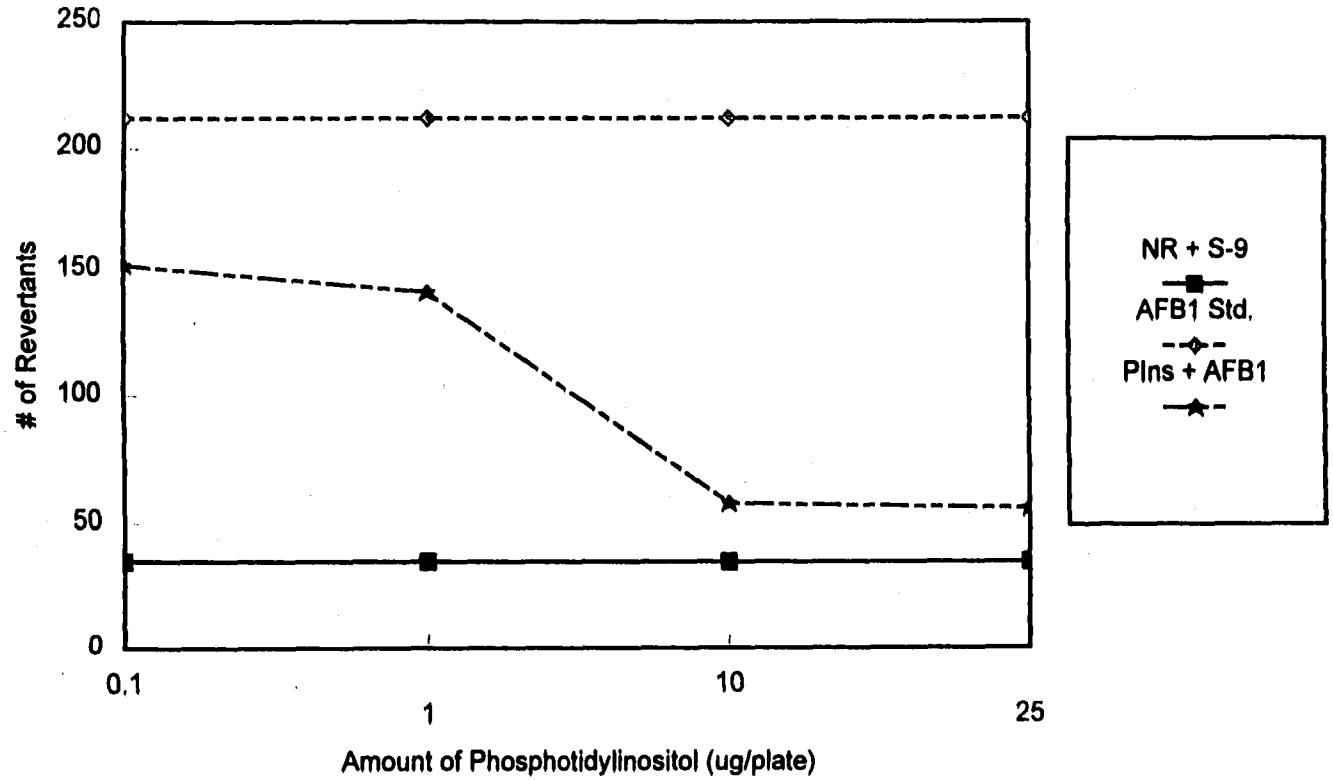

Figure 3.14 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA98) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (25 ng/plate) in DMSO. Values are mean +/- standard error of three replications.

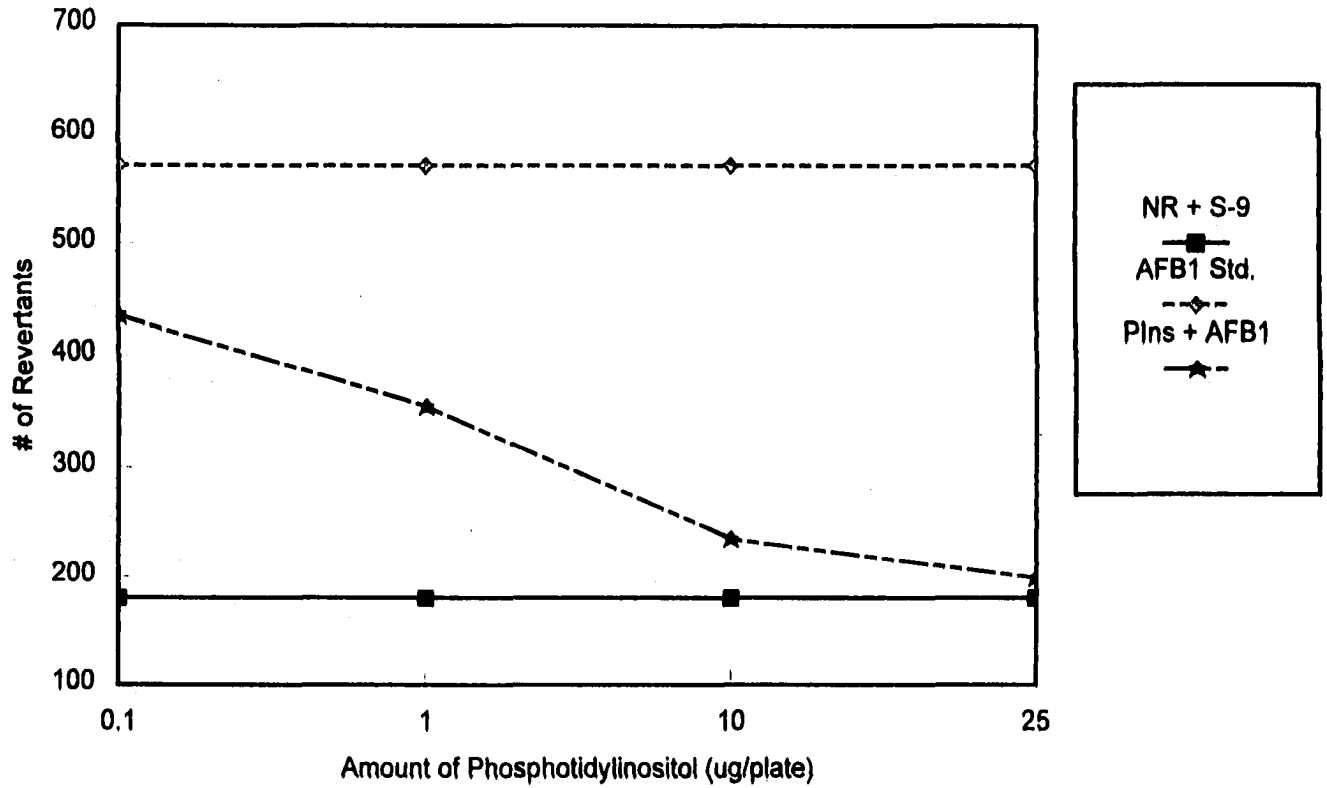

Figure 3.15 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA-100) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (25 ng/plate) in DMSO. Values are mean +/- standard error of three replications.

revertants decreases and there was no mutagenicity of AFB_1 at the concentration of 25 μg/plate. With the other indirect-acting mutagen studied, i.e., 2-AF, the antimutagenic potential was not significantly different from the control (Figures 3.16 and 3.17), irrespective of both tester strains and concentration of phosphotidylinositol. However, the reduction in the number of revertants was ~ 45% regardless of tester strain. It may have been because of the different mode of action of the two mutagens. It has been observed that 2-AF does not form an epoxide like AFB_1 to render it mutagenic for interaction with DNA adducts.

The antimutagenic potential of phosphotidylinositol was also tested against the direct-acting MNNG and NaN_3. However, it was not found to be antimutagenic against these mutagens (Figures 3.18 and 3.19) in tester strain TA-100. The reduction in the number of revertants was 50% in the case of MNNG and ~25% against NaN_3. It is important to note that the amount of phosphotidylinositol was less than the amount tested against other mutagens. Further investigation with a higher level of phosphotidylinositol is warranted.

The data of this study show that phosphotidylinositol was significantly anti-mutagenic against the indirect-acting AFB_1 and partially against 2-AF. With regard to direct-acting MNNG and NaN_3, the antimutagenicity was not significant; however, there was a 50% and 35% reduction in the number of revertants as evident against MNNG and NaN_3, respectively. This data also suggests an indirect role of this anti-mutagenic compound. It is a possibility that phosphotidylinositol might have inhibited the metabolic activation of both

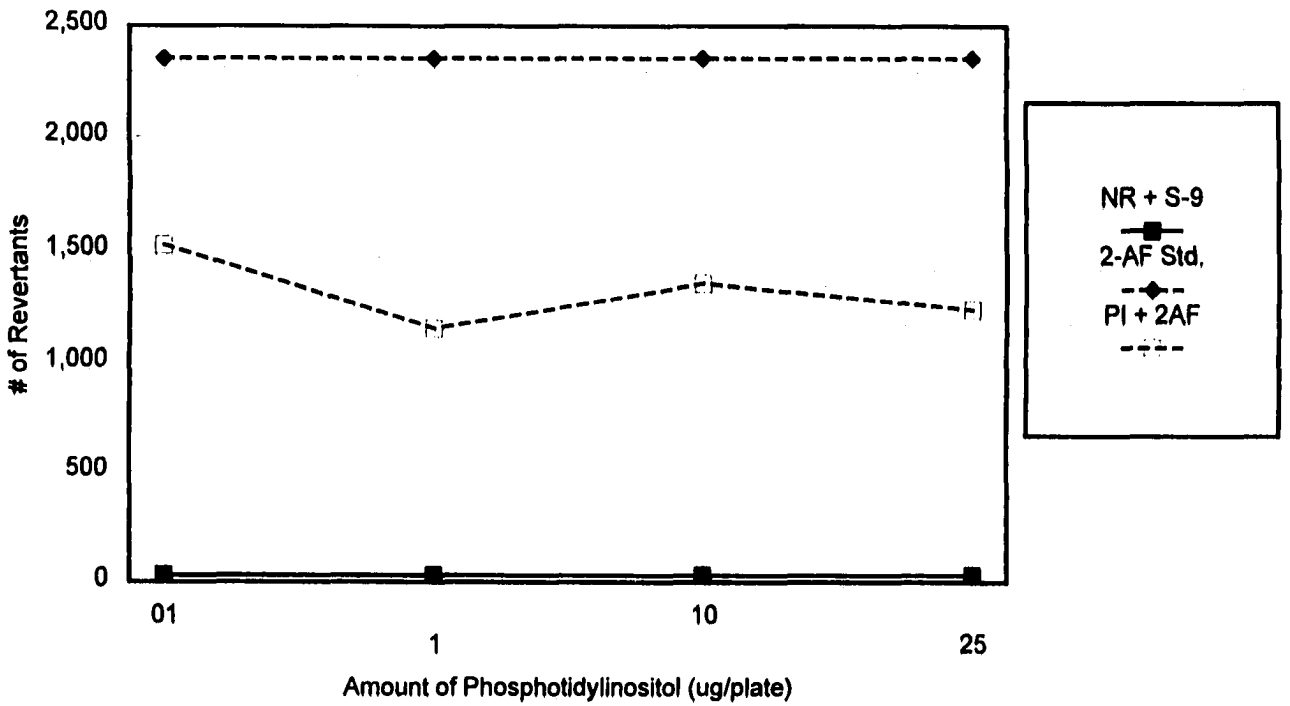

Figure 3.16 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA-98) with metabolic activation (S-9) against 2-AF. 2AF=2-aminofluorine (5 ug/plate) in DMSO. Values are mean +/- standard error of three replications.

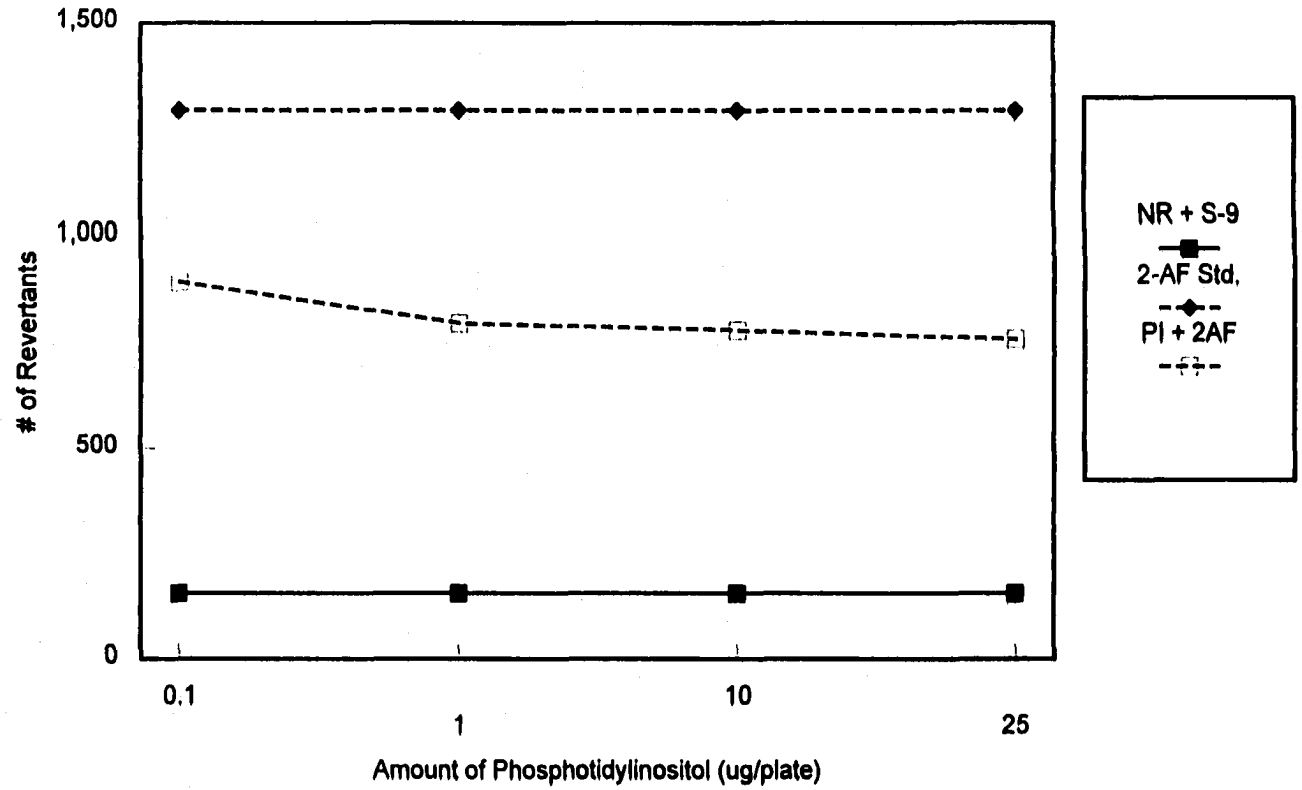

Figure 3.17 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA-100) with metabolic activation (S-9) against 2-AF. 2AF= 2-aminofluorine (5ug/plate) in DMSO. Values are mean +/- standard error of three replications.

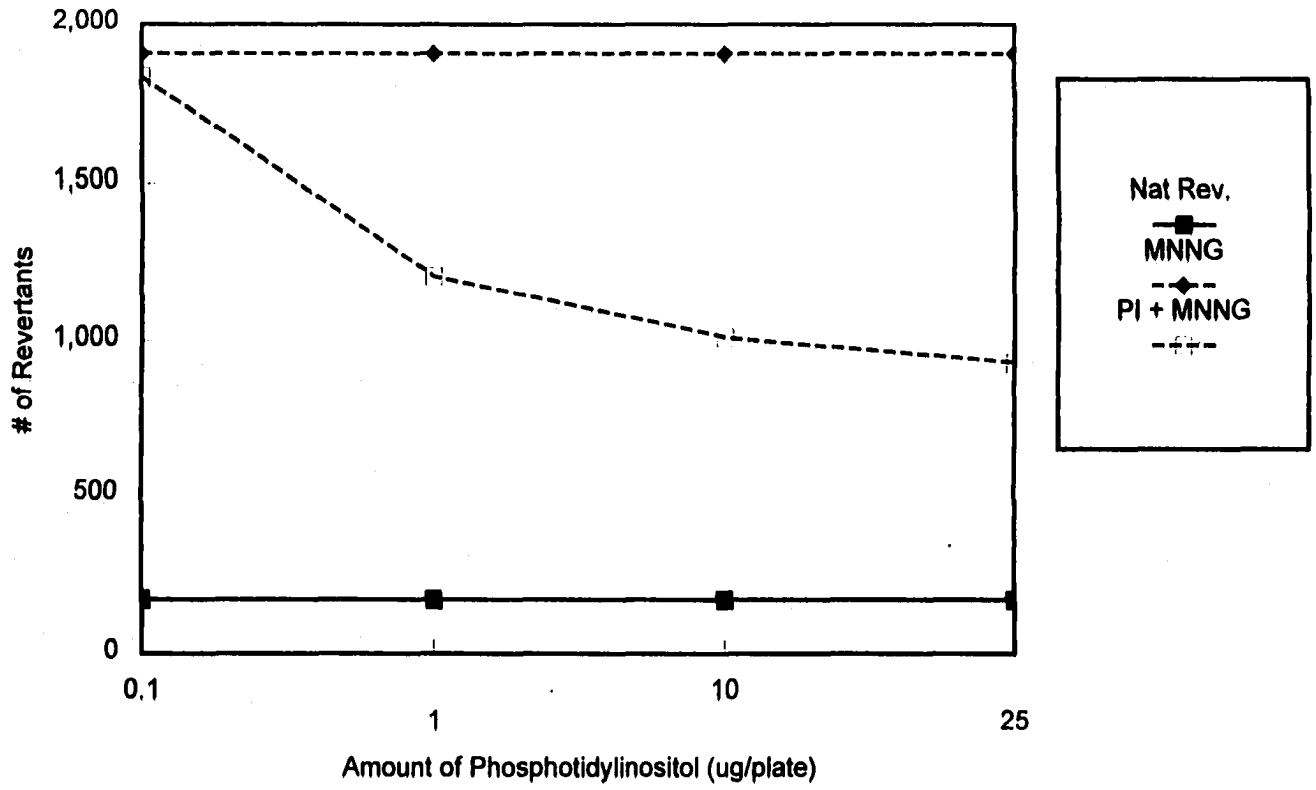

Figure 3.18 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA100) without metabolic activation (S-9) against MNNG. MNNG=methyl-nitro-nitrosoguanidine (1ug/plate) in DMSO. Values are mean +/- standard error of three replications.

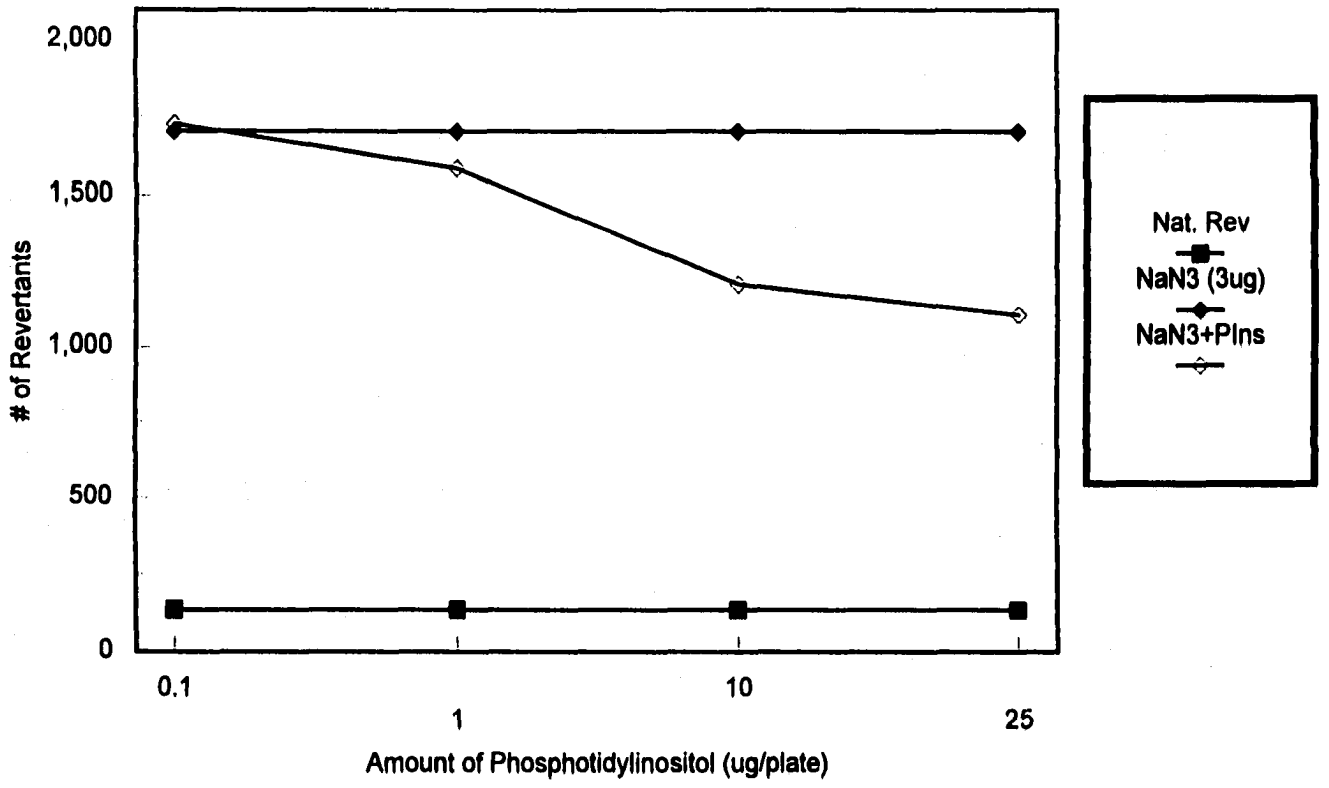

Figure 3.19 Mutagenic potential of phosphotidylinositol in *Salmonella* microsomal mutagenicity assay (TA100) without metabolic activation (S-9) against NaN_3. NaN3=sodium azide (1ug/plate) in water. Values are mean +/- standard error of three replications.

indirect-acting mutagens. It has been reported previously that phosphotidyl-inositol inactivates the enzymes required for metabolic activation through its phosphorylation products (Eaton and Graf, 1990). Furthermore, PI phosphorylates both lower and higher inositol phosphates and other by-products, such as polyunsaturated fatty acids and diacylglycerol. Linoleic acid and oleic acid have been shown to inhibit the mutagenicity of different compounds *in vitro* (Aikawa nd Komatsu, 1987; results of next trial). Since phosphorylation results in the production of other anti-mutagenic factors, it is also possible that the antimutagenicity was due to the production of other compounds besides the already tested linoleic acid.

Although literature has reported its role at various stages during plant growth, no previous report was found regarding the anti-mutagenic potential of phosphotidylinositol by itself. However, the by-products of its phosphorylation /metabolism have been reported anti-mutagenic/anti-carcinogenic both *in vivo* and *in vitro* (Shamsuddin, 1995). The results of this study are consistent with the previous studies (Aikawa, 1988; Ho *et al.*, 1992; and Aikawa and Kamatsu, 1987; Parodi, 1997), which have reported the anti-mutagenic potential of polyunsaturated fatty acids and their by-products.

Therefore, it is difficult to suggest on the basis of these results whether phosphotidylinositol itself is anti-mutagenic or is anti-mutagenic through its phosphorylation products. For the purpose of investigating this hypothesis, the anti-mutagenic potential of linoleic acid has also been investigated.

3. Anti-mutagenic Potential of Linoleic Acid Against Direct-acting and Indirect-acting Mutagens

Linoleic acid at different concentrations was tested against the mutagenic activity of direct- and indirect-acting mutagens. Linoleic acid is a poly-unsaturated fatty acid and it is attached to the glucosidic ring structure of *myo*-inositol through a phosphodiester bond forming a phosphotidylinositol molecule, a substrate for the phosphorylation of phytic acid.

Linoleic acid completely inhibited the mutagenicity of AFB_1 at a concentration of 10μg/plate in both tester strains (TA-98 and TA-100) (Figures 3.20 and 3.21). However, it showed a slight cytotoxicity at higher concentrations (100 and 1000μg/plate) in the Ames *Salmonella* mutagenicity assay. While studying the mutagenic potential of aflatoxin/ammoniation products, Weng *et al.* (1997) reported that corn containing high concentrations of AFB_1(7500 ppb) was not mutagenic in the Ames *Salmonella* microsomal mutagenicity assay. They suggested the presence of "unknown interfering materials" that had properties to inhibit the mutagenic potential of AFB_1. Later, these unidentified "unknown interfering materials" were identified and characterized as linoleic acid-like compounds along with AFB_1 in the same bands of thin-layer chromatographic (TLC) plates in different varieties of corn (Burgos-Hernandez, 1998). The results of the current study suggest the antimutagenic role of linoleic acid against AFB_1 in the Ames *Salmonella* microsomal mutagenicity assay.

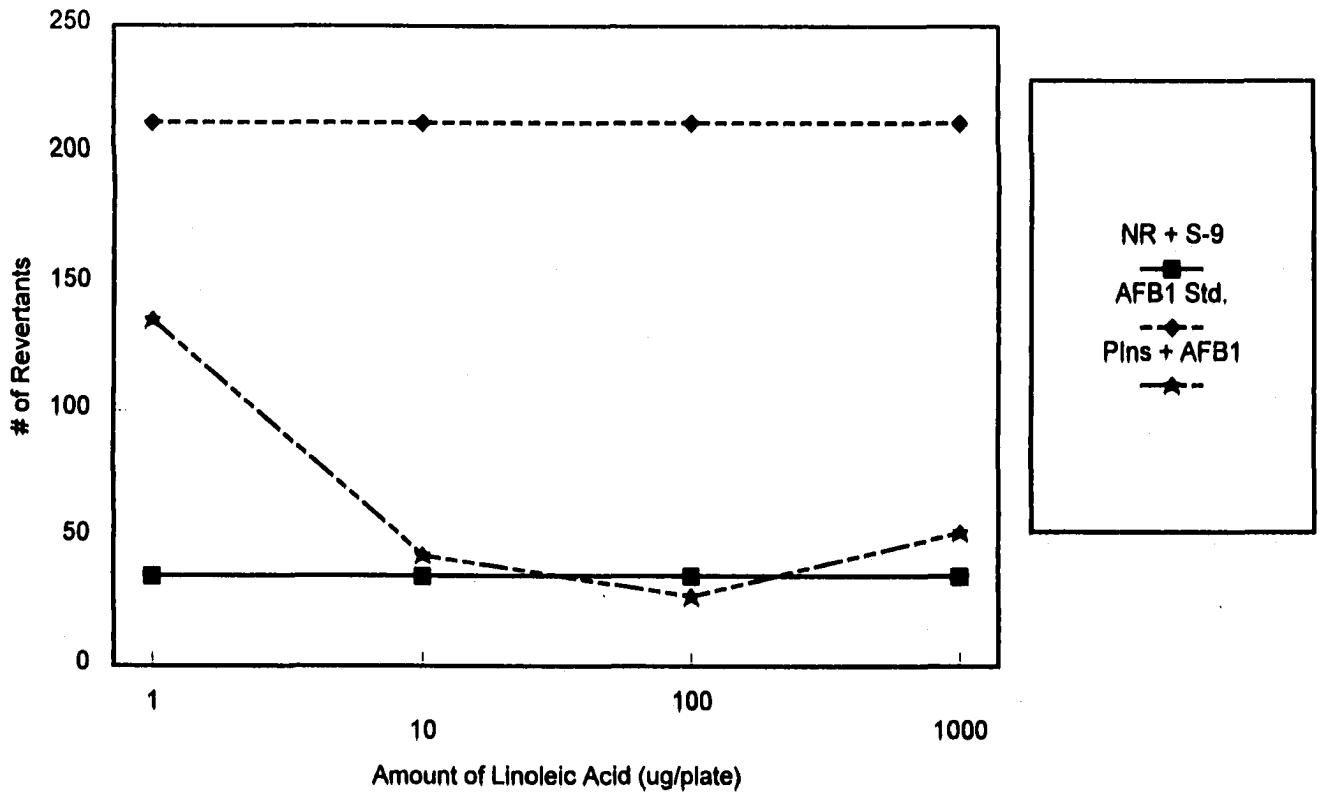

Figure 3.20 Mutagenic potential of linoleic acid in *Salmonella* microsomal mutagenicity assay (TA98) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (25 ng/plate) in DMSO. Values are mean +/- standard error of three replications.

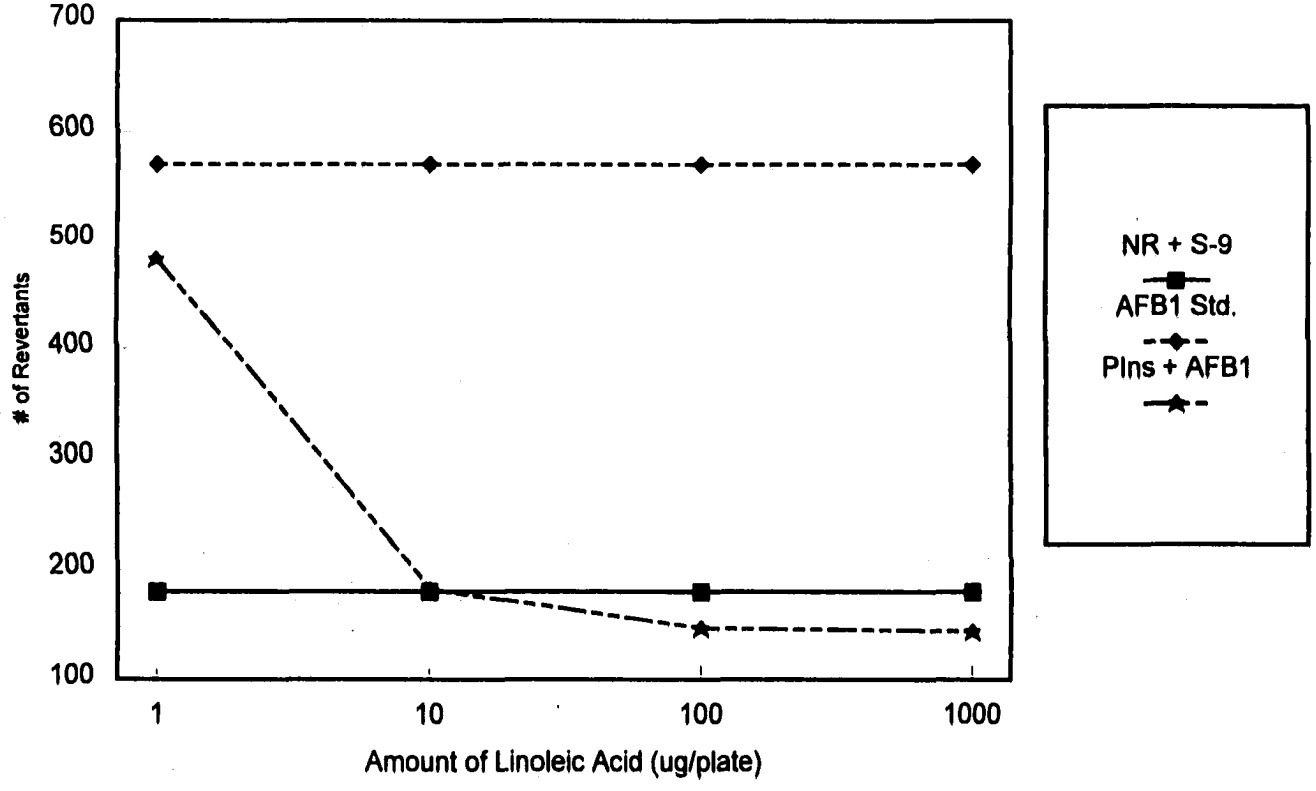

Figure 3.21 Mutagenic potential of linoleic acid in *Salmonella* microsomal mutagenicity assay (TA100) with metabolic activation (S-9) against AFB_1. AFB_1=aflatoxin B_1 (25 ng/plate) in DMSO. Values are mean +/- standard error of three replications.

The results from the other indirect-acting 2-AF trial did not show any antimutagenicity at 10 μg/plate; however, 100 μg/plate of linoleic acid was antimutagenic against 2-AF in both tester strains (Figures 3.22 and 3.23). It has been reported previously (Aikawa, 1988) that conjugated linoleic acid was inhibitory to the mutagenicity of 2-AF in the *Salmonella* microsomal mutagenicity assay with tester strain TA-98 with metabolic activation and the inhibition of mutagenicity was a function of increased concentration of linoleic acid. The results of this trial confirm the previous reports.

Similar results were also achieved against direct-acting MNNG and NaN_3. However, the amount of linoleic acid was higher (>100μg/plate) to obtain the same effect on these mutagens (Figures 3.24 and 3.25). Since direct-acting mutagens do not require metabolizing enzymes (S-9), the data suggest the direct interaction of linoleic acid with the mutagenic compounds.

Since AFB_1 and 2-AF both require microsomal fractions (S-9) for the metabolic activation of these compounds prior to exhibiting mutagenic potential, the role played by linoleic acid as an antioxidant for the tester strains. There might have been a probable direct interaction of linoleic acid with the mutagens, or through interaction with enzymes present in the fraction that could inhibit the transformation of AFB_1 to the reactive metabolite, AFB_1-8,9-epoxide. However, the results do not permit the determination of whether linoleic acid was acting directly on the reactive metabolite or on the metabolizing enzymes (S-9). But on the basis of comparison between the anti-mutagenicity of linoleic acid against

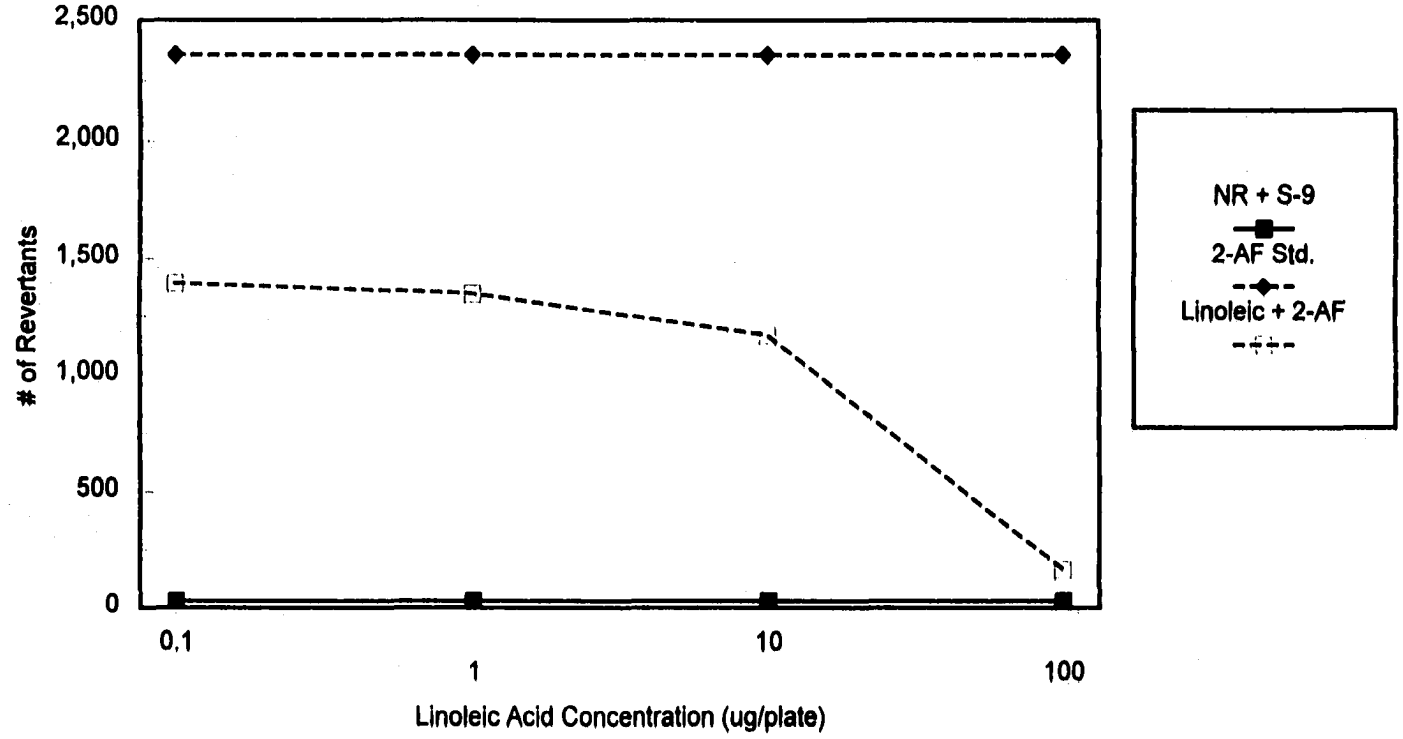

Figure 3.22 Mutagenic potential of linoleic acid in *Salmonella* mutagenicity assay (TA98) with metabolic activation (S-9) against 2-AF. 2AF= 2-aminofluorine (5ug/plate) in DMSO. Values are mean +/- standard error of three replications.

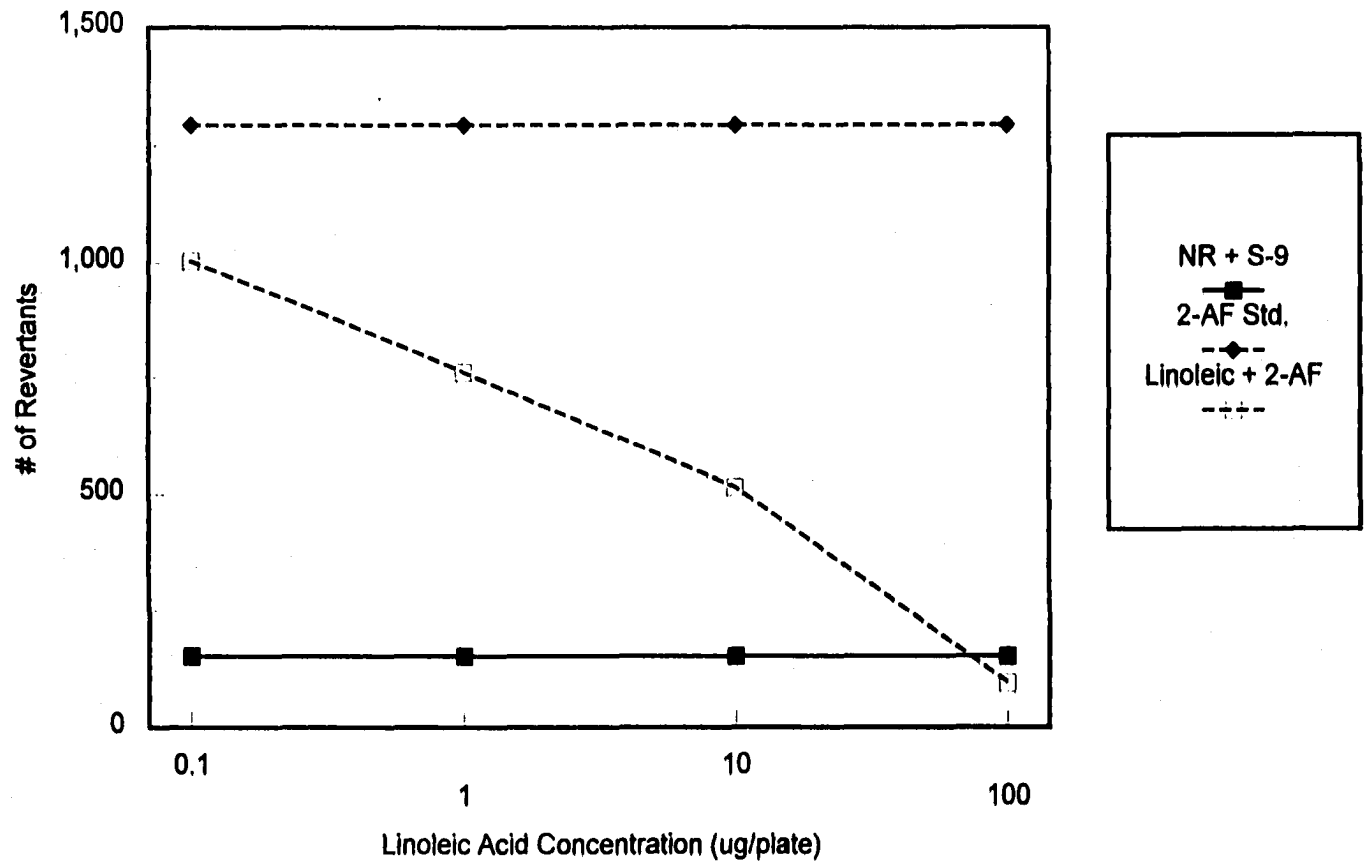

Figure 3.23 Mutagenic potential of linoleic acid in *Salmonella* microsomal mutagenicity assay (TA100) with metabolic activation (S-9) against 2-AF. 2AF= 2-aminofluorine (5ug/plate) in DMSO. Values are mean +/- standard error of three replications.

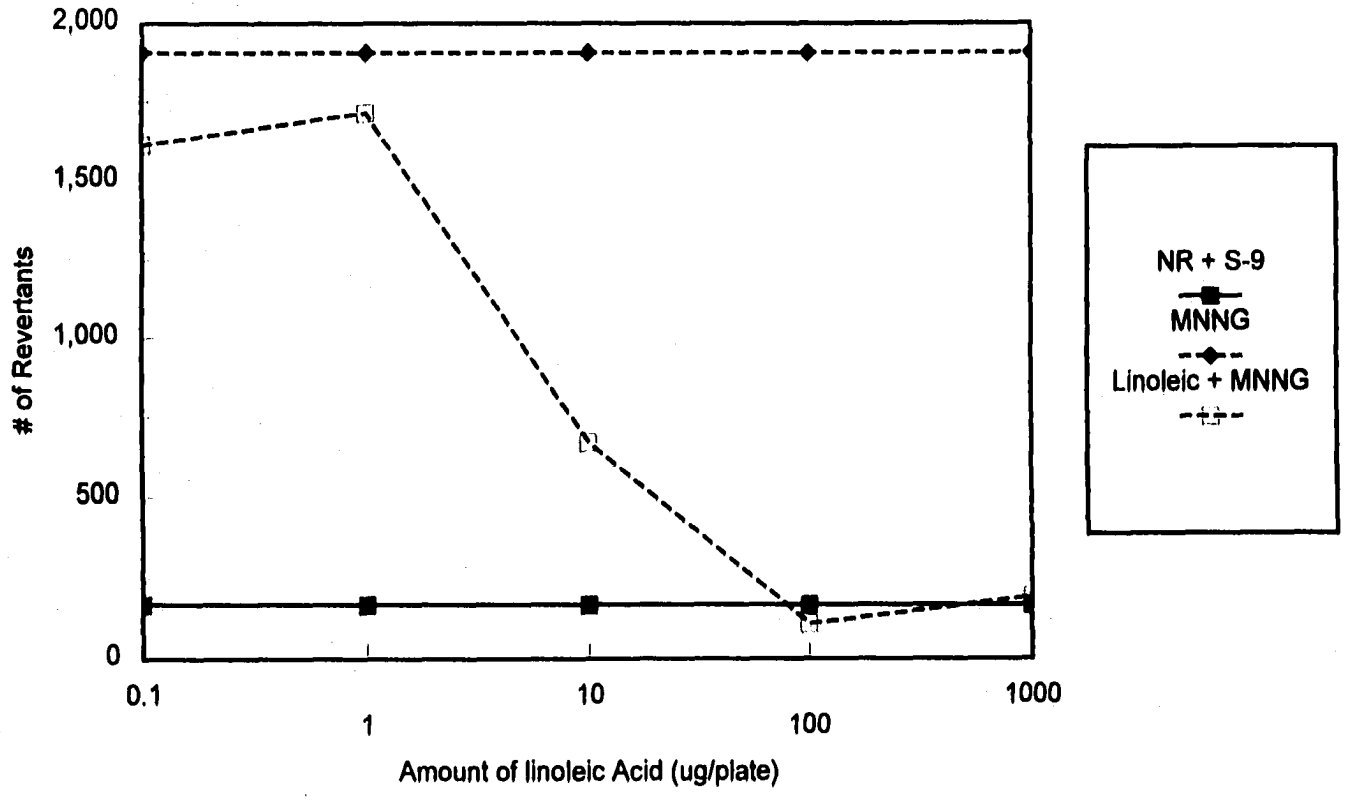

Figure 3.24 Mutagenic potential of linoleic acid in *Salmonella* microsomal mutagenicity assay (TA100) without metabolic activation (S-9) against MNNG. MNNG=methyl-nitro-nitrosoguanidine (1ug/plate) in DMSO. Values are mean +/- standard error of three replications.

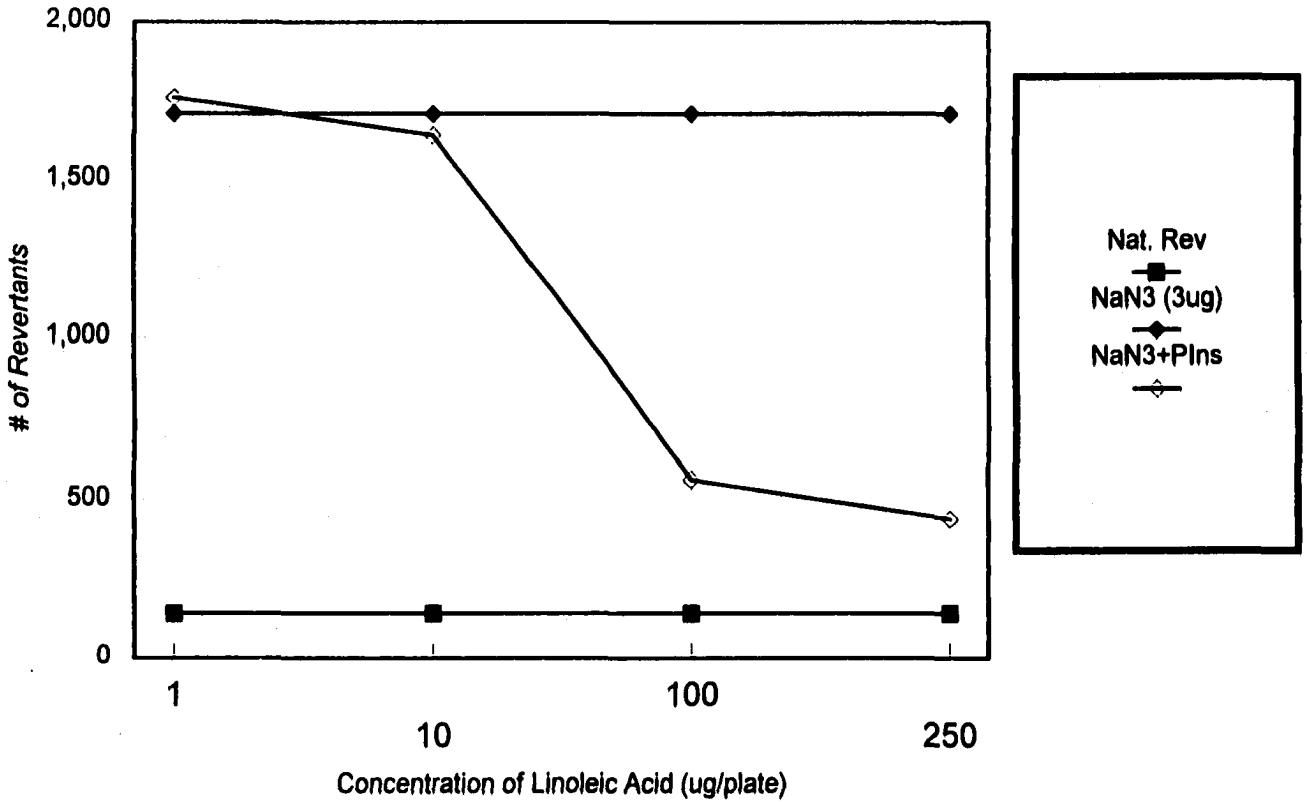

Figure 3.25 Mutagenic potential of linoleic acid in *Salmonella* microsomal mutagenicity assay (TA100) without metabolic activation (S-9) against NaN_3. NaN3=sodium azide (1ug/plate) in water. Values are mean +/- standard error of three replications.

indirect-acting mutagens with direct-acting mutagens, the data suggest a stronger possibility of a direct interaction with mutagens or formation of a complex between linoleic acid and mutagens. These results are consistent with the previous reports regarding the antimutagenic potential of linoleic acid against *uv*-induced mutagenesis in *Escherichia coli* (Aikawa and Komatsu, 1987), and 2-aminofluorene in Ames *Salmonella* microsomal mutagenicity assay (Aikawa, 1988). These results also confirms that the "unidentified factors" in corn reported previously as linoleic acid-like compounds (Burgos-Hernandez, 1998; Huang *et al.*, 1997; Weng *et al.*, 1997)

Furthermore, it is also possible that other nutrients present in the media could interact and play a role in concert to render this antimutagenicity. The role of linoleic acid was also tested in Ames *Salmonella* mutagenicity assay because of the fact that it may have more antimutagenic properties than phosphotidylinositol. However, both compounds showed similar antimutagenic potential against direct- and indirect-acting mutagens in the Ames *Salmonella* mutagenicity assay regardless of tester strains tested.

D. Summary

Non-nutritive components present in food are thought to play an important role in the reduction of diet-related cancers. Production of mycotoxins, especially aflatoxin B_1 (AFB_1), in cereals is correlated with a higher incidence of liver cancer throughout the world. The presence and search for known and unknown antimutagens/anticarcinogens in plant food has been reported extensively. AFB_1, when extracted from the meal matrix, shows a lower

mutagenic potential than the pure AFB_1. Inositol phosphates (InsPs) are presumed to be one of these intrinsic components since their presence account for more than 1% (on dry wt. basis) in cereals, oilseeds and nuts. InsPs have been involved in signal transduction and cell to cell communication. The presence of this compound warrants investigation against mutagens present in the human environment.

The objective of this study was to evaluate the antimutagenic properties of inositol phosphates, linoleic acid and phosphotidylinositol against indirect-acting (Aflatoxin B_1 [AFB_1] & 2 aminofluorene [2-AF]) and direct-acting (sodium azide [NaN_3] & methyl nitro-*N*- nitrosoguanidine[MNNG]) in the Ames *Salmonella* microsomal mutagenicity assay. A standard plate incorporation procedure of Ames assay was used by using tester strains TA100 and TA98. AFB_1, 2-AF, MNNG in dimethyl sulfoxide (DMSO) and NaN_3 in water were used as standard mutagens/ carcinogens and tested against various salts of phytic acid, linoleic acid, and phosphotidylinositol at different concentrations.

A reduction in the number of revertants was shown to be a function of increased concentration of phytic acid in *Salmonella*/microsomal mutagenicity assay. Calcium, magnecium, potassium & sodium salts of phytic acid and *myo*-inositol substantially reduced the number of revertants irrespective of mutagen type. The largest reduction (20-50%) was observed against direct-acting NaN_3, i.e., calcium-salt (10-55%), sodium-salt (10-20%) and *myo*-inositol (20-35%). Against AFB_1, the reduction was: calcium-salt (20%), potassium-salt (20-50%), magnecium-salt (10-30%), and *myo*-inositol (20-40%). Similar results were

observed with MNNG and 2-AF in the same assay. However, the results of the phytic acid trial do not suggest it as a complete anti-mutagenic compound in the Ames test. This was the first study regarding the evaluation of phytic acid and its various salts in *Salmonella/* Microsomal Mutagenicity Assay. Further investigations on the role of phytic acid are warranted *in vivo* to evaluate its potential anti-mutagenic properties against aflatoxin B_1 mutagenicity.

On the other hand linoleic acid and phosphotidylinositol showed a more promising antimutagenic role in this assay against most of the mutagens in both tester strains (TA-98 and TA-100). Linoleic acid has previously been shown anti-mutagenic both *in vivo* and *in vitro*. The potential role for this compound in mutagenesis should also be investigated against indirect-acting aflatoxin B_1 *in vivo*.

These results also suggest that if phytic acid and linoleic acid are made available in the diet by introducing more fibrous portions of cereals, it can protect against carcinogenesis.

4. EVALUATION OF THE ANTI-MUTAGENIC PROPERTIES OF PHYTIC ACID AGAINST THE PRODUCTION OF AFLATOXIN BY *ASPERGILLUS FLAVUS* (LINK EX. FRIES) IN CZAPEK-DOX LIQUID MEDIUM

A. Introduction

Phytic acid, *myo*-inositol hexaphosphoric acid, is widely present in cereals, oil seeds, nuts and legumes, especially in their shells, buddings and outer cotyledons. The function of phytic acid is to provide cations (Milliams, 1970), phosphorous (Hall and Hodges, 1966, Eaton and Graf, 1985), or high-energy phosphoric groups (Biswas *et al.*, 1978) necessary for the early growth and development of plants. Phytic acid possesses significant antioxidant (Graf *et al.*, 1987) and antineoplastic (Shamauddin, 1995) potentials. The chemopreventive potential of phytic acid has been observed in the experimental models of colon (Ullah and Shamsuddin, 1990), liver (Hirose *et al.*, 1991) and mammary (Vucenik *et al.*, 1995) carcinogenesis.

Aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus* as well as other fungi is an intermediate metabolite of glucose aerobic oxidation (Dayi *et al.*, 1995), and enzymes that require metal ions for activation play an important role for AFB_1 production. There is a lot of evidence that AFB_1 causes primary liver cancer (PLC) in human and other neoplasms in animals. As a result the growth of mycotoxins producing fungi cause an estimated loss of more than $200 billion in both food and feeds and other industrial materials (Dayi *et al.*, 1995). Various studies *in vivo* have shown the antimutagenic potential of phytic acid. This study was conducted to evaluate the efficacy of phytic acid in preventing

the production of AFB_1 by *Aspergillus flavus* in Czapak-Dox liquid media. Czapek-Dox medium is a synthetic liquid medium. It contains different metal ions that are required for the growth and metabolism of selective molds.

The objective of this study was to evaluate the anti-aflatoxin/anti-mutagenic potential of phytic acid in a synthetic liquid medium and the factors that govern its chelating properties on the biosynthesis of aflatoxins by *Aspergillus flavus*.

B. Materials and Methods

1. Corn and Fungal Cultures

Non-contaminated whole kernel corn samples were obtained from Cargill Co. (Port Allen, LA). *Aspergillus flavus* (Link ex. Fries) was kindly provided by Dr. Kanneth Damann (Department of Plant Pathology, Louisiana State University, Baton Rouge, LA).

2. Chemicals

Cupric sulfate, dipotassium hydrogen phosphate, ferrous sulfate, magnesium sulfate, sodium nitrate, zinc sulfate were purchased from Sigma Chemical Co. (St. Louis, MI). Potato dextrose agar was obtained from Difco Laboratories (Detroit, MI) and glucose (dextrose) and sucrose from Fischer Scientific Co. (Fair Lawn, NJ). Chloroform (HPLC grade), ethyl ether anhydrous, hexane (HPLC grade), methanol (HPLC grade), potassium chloride, potassium phosphate dibasic anhydrous, and water (HPLC grade) were acquired from Mallinckrodt Baker Inc. (Paris, KT). Thin layer chromatography plates were obtained from Sigma Chemical Co. (St. Louis, MI).

Pure Aflatoxin B_1 standard was generously provided by Dr. Mary S. Trucksess (CFSAN-FDA, Washington, D.C.). Aflatoxin B_1, B_2, G_1, and G_2 HPLC standards (50 μg/g) mixture was purchased from Romer Laboratories Inc. (Union, MO).

3. Fungal Growth and Harvesting Culture Inoculum

Fungi (*A. flavus*) was cultured on potato dextrose agar (PDA). In order to prepare the PDA broth medium, 39 g of potato dextrose agar was dissolved in 1L of sterile distilled water and was autoclaved at 121° C for 30 min. After the broth temperature reached about 45° C, the medium was aseptically poured on the sterile petri dishes (15 x 25 mm). The plates were seeded with *Aspergillus flavus* (AF-13 Link ex. Fries) plugs under the laminar hood and grown for 7 days at 30° C. Cultured material was scraped off and rinsed with sterile distilled water (containing 0.1% Tween-20) (ca. 10 ml/plate). The mold/water suspension from each plate was collected to make a stock of inoculating conidial suspension. The concentration of stock inoculating conidial solution was calculated by using a hemacytometer (model: Ortholux II, American Optical Co. Buffalo, NY). For each experiment, a conidial suspension of 10^7 conidia/ml was prepared by diluting the stock conidial solution in sterile distilled water.

4. Safety precautions

Since a highly toxigenic strain of *Aspergillus* was used in this study, several safety measures were required to avoid exposure to toxic material and mold spores. A half-face mask respirator equipped with HEPA filters was used

every time the corn samples were handled. Also, protective eye wear and nitrile gloves were worn at all times. All samples were handled aseptically in a biological laminar flow hood. It is important to take maximum safety precautions during the inoculation study and the toxin extraction, as high sporulation leads to a high volatility of spores. Each and every area including the hood should be thoroughly sterilized with 95% ethanol.

5. Preparation of Czapek-Dox Medium

a. Phytic acid trial

Czapek-Dox medium was prepared with slight modifications from the procedures presented in the literature (Hamid and Smith, 1987; Dayi *et al.*, 1995). Seventy grams of clean corn powder were weighed in a 1000 ml beaker and 500 ml of distilled water was added. The mixture was brought to boil and filtered through a Whatman # 4 filter paper into 1000 ml flask. This was followed by dissolving/adding 30 g of sucrose, 3 g $NaNO_3$, 1 g K_3HPO_3, 0.5 g K_3Cl_3, 0.5 g Mg SO_4, 1 ml of 1% $FeSO_4$, 0.5% $CuSO_4$, and 1% $ZnSO_4$ each over a hot plate. The volume was brought to 100 ml by adding distilled water and was distributed in ten 100 ml flasks. The flasks were stoppered and sterilized for 20 min at 121° C.

The effect of phytic acid concentration (Table 5.1) and Aflatoxin B_1 production was tested by adding 1 ml of *Aspergillus flavus* suspension (10^7 conidia) per flask. The production of Aflatoxin B_1 was monitored by determining

the amount of AFB_1 after 5, 10, 15, and 25 days incubation of the samples at 28° C on a rotatory shaker.

Table 4.1 Experimental protocol for phytic acid concentration trial on AFB_1 in Czapek-Dox liquid medium

Treatment	Culture Number/Treatments A	B	C	D	E	F	G
Phytic Acid (mg/100ml)	0	0.01	0.05	0.10	0.20	0.50	1

b. Metal ions trial

Czapek-Dox liquid agar was prepared as described previously in the literature (Dayi *et al.*, 1995; Hamid and Smith, 1987; Marsh, 1975; Montville and Goldstein, 1989; Montville and Goldstein, 1987) with modifications. Briefly, 70 grams of clean corn powder were weighed in a 1000 ml beaker and 500ml of distilled water were added. The mixture was brought to a boil and filtered through a Whatman # 4 filter paper into a 1000 ml flask. It was followed by adding 30 g of sucrose, 3 g $NaNO_3$, 1 g K_3HPO_3, 0.5 g K_2Cl_3, 0.5 g $MgSO_4$ (all except in culture # F), 1 ml each of 1% $FeSO_4$ (all except in culture # E), 0.5% $CuSO_4$ (all except in culture # D) and 1% $ZnSO_4$ (all except culture # G) over a hot plate. The volume was brought to 1000 ml by adding distilled water and the volume was distributed in ten 100 ml conical flasks. The flasks were stoppered and sterilized for 20 min at 121° C.

The effect of metal ions on the production of AFB_1 (Table 5.2) was tested by adding 1 ml of *Aspergillus flavus* suspension (10^7 conidia) per flask. The production of AFB_1 was monitored by determining the amount of AFB_1 after 5, 10, and 15 days incubation of the samples at 28° C on a rotatory shaker.

Table 4.2 Experimental protocol for metal ions trial on production of AFB_1 from *A. flavus* in Czapek-Dox liquid medium

Treatment	Culture Number/Treatments A	B	C	D	E	F	G
Phytic Acid (mg/100ml)	0	0.1	1	0.1	0.1	0.1	0.1

Where D=without $CuSO_4$ E=without $FeSO_4$
F=without $MgSO_4$ G=without $ZnSO_4$

6. Extraction of Aflatoxin

Briefly, 20 ml of the collected culture samples were diluted with the addition of methanol: water: hexane (55:45:30) in a 250 ml flask and stoppered to avoid leakage. The flasks were shaken for 30 min. on a rotary shaker. The mixture was filtered through a Whatman # 4 filter paper into a separating funnel for layering. The MeOH:H_2O extract was collected in a conical flask. Twenty ml of this extract were pipetted into another separating funnel and 20 ml of chloroform was added to each sample. The samples were rigorously shaken for 2 min. and left to stand for a few minutes for layering. The lower portion of chloroform was collected in scanillating vials. The chloroform extracts were

considered the raw AFB_1 samples. The extracts were dried under nitrogen and reconstituted in 1 ml chloroform and spotted on TLC plates. Figure 4.1 depicts the flow diagram of this experiment.

7. Thin-layer Chromatography (TLC)

TLC plates (20 x 20 cm with 1 mm thick selica gel) obtained from Sigma-Aldrich (St. Louis, MO) were used to determine the amount of aflatoxins. Thin layer chromatography was performed in two developing chambers (25 x 10 x 8 cm) at room temperature with modifications according to the method reported by Dayi *et al.*, 1995.

Briefly, 10 µl portions of the samples were applied on the point of origin (2 cm) apart from the lower edge of the TLC plate, and the TLC plates were placed in the first developing chamber containing 100ml absolute diethyl ether as mobile phase. The plates were removed after the moving edge of ether reached about 10 cm beyond the point of origin. The solvent front was marked and the plates were dried under the laminar hood and were placed in the second chamber containing 100 ml chloroform: acetone (9:1) as mobile phase. The plates were removed from the chamber after the mobile solvent reached 10 cm beyond the point of origin or the solvent front mark. Plates were allowed to dry under the laminar hood. Since no fats were present in the samples, ethyl ether was not used in the 10, 15, and 25 day samples. AFB_1 and AFB_2 were collectively detected from the samples on TLC plates by comparing them with

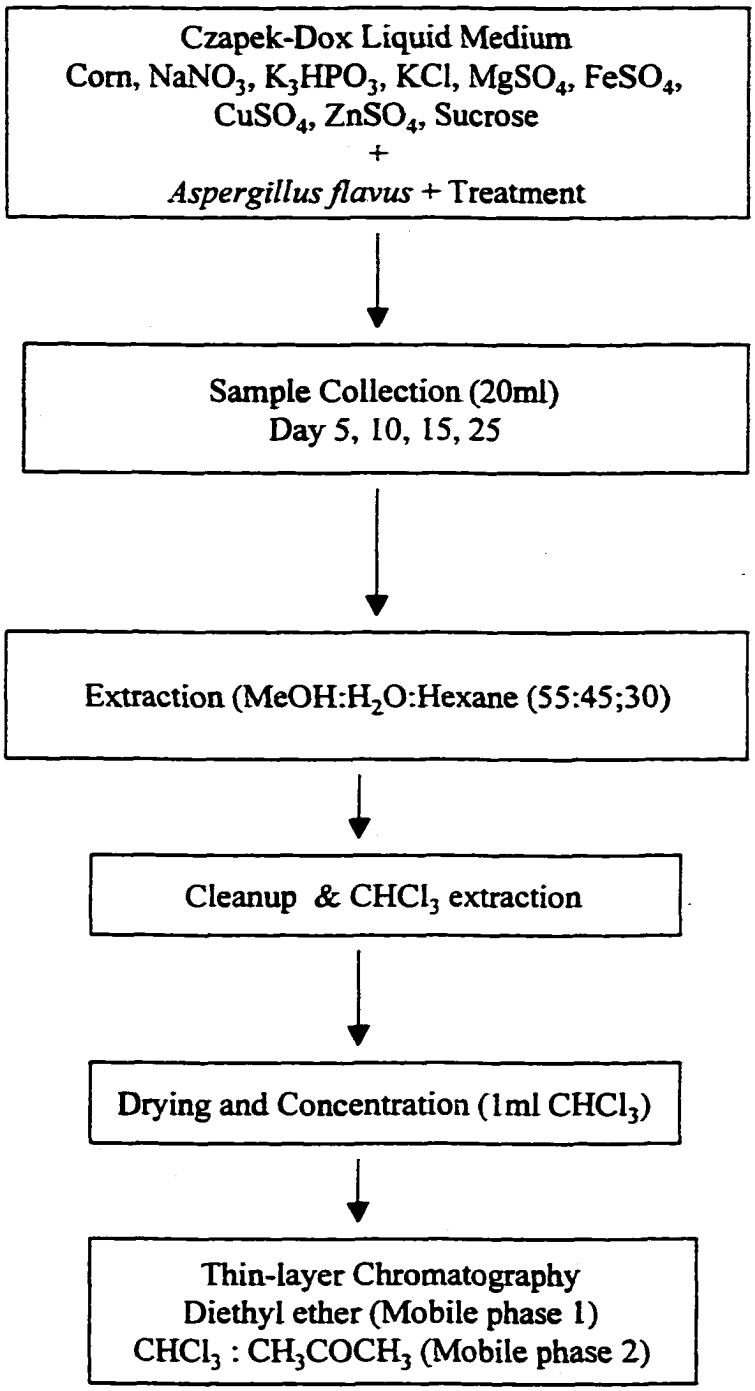

Figure 4.1 Flow diagram for the preparation of Czapek-Dox liquid medium.

AFB_t standards. Fluorescence intensity of AFB_t spots was measured at 365 nm UV light. *Rf*-value of each spot was measured.

8. Statistical Analysis

One way analysis of variance (ANOVA) was used to determine the difference in toxin production in different treatments of both trials. Statistical Analysis Systems (SAS) was used to conduct the statistical analysis (SAS, 1988). Scheffe's test with ($P<0.05$) was used to compare the difference among different treatments.

C. Results and Discussion

1. Effect of Phytic Acid Concentration on Aflatoxin Production

Inhibition of aflatoxin formation was found to be a function of an increase in phytic acid concentration (Appendix A). Results of this study suggest that phytic acid at a concentration of 0.5% or more in the culture can inhibit the synthesis of AFB_t by *A. flavus*.

According to the results, *A. flavus* was able to synthesize aflatoxin throughout the duration of study in the control treatment (A) (Figure 4.2). Although in treatments B and C, the level of phytic acid added did not inhibit the total production, the production of aflatoxin was lower than the control treatment (A). Treatments D - E, where the amount of phytic acid was less than 0.5 mg/100 ml of the medium, in the first two sets of samples at day 5 and 10, not a significant amount of aflatoxin was produced. No detectable production of aflatoxin was achieved in treatments F and G. These results confirm that the synthesis of aflatoxin can be controlled by adding phytic acid in the liquid

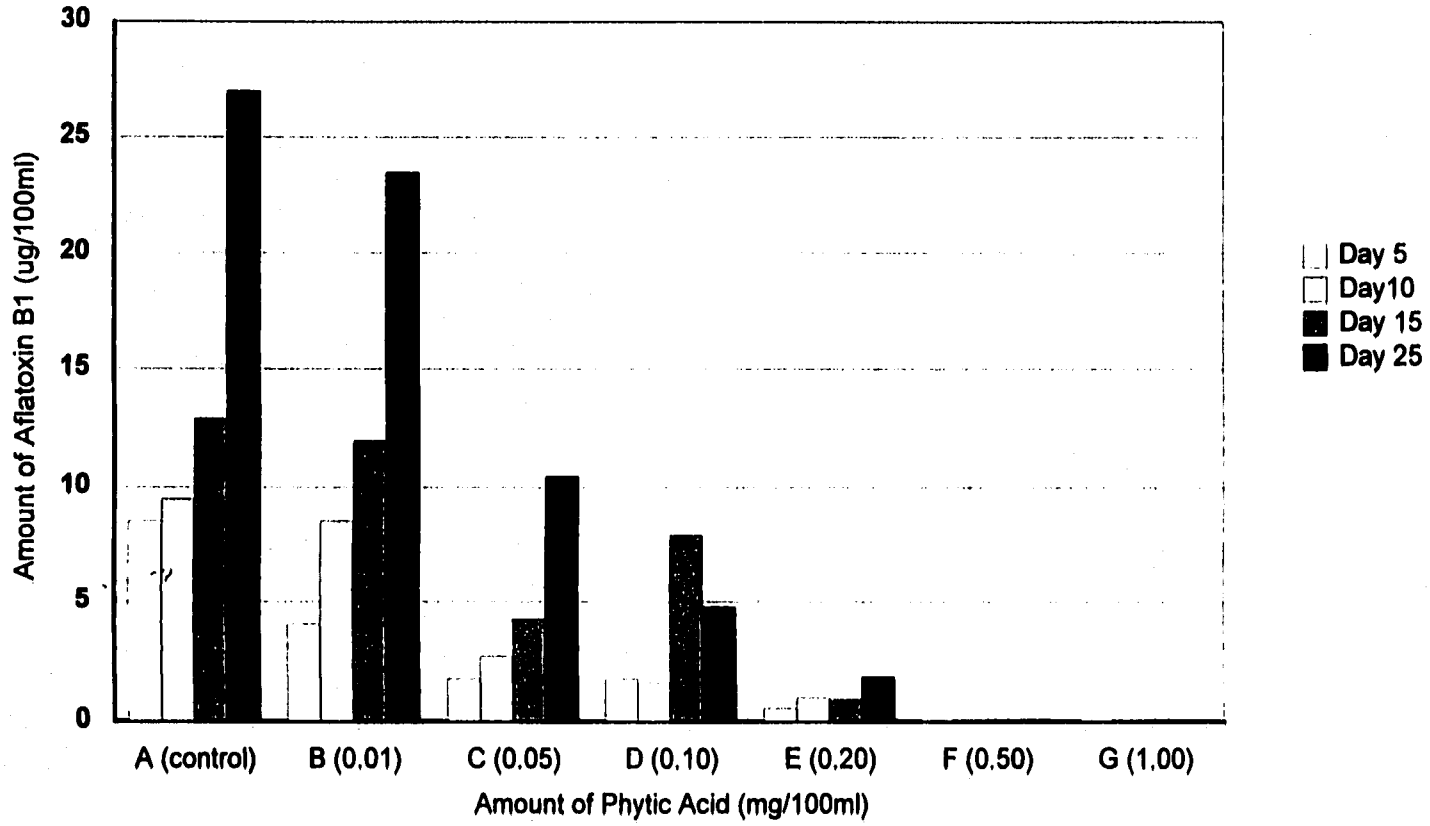

Figure 4.2 Effect of phytic acid concentration on the production of AFB_1 from *A. flavus* in Czapek-Dox liquid medium. Values are mean +/- standard error of three replications.

media. The gradual process of inhibition began in treatments C-E, and continued until the end of the study period in these treatment sets. The optimum amount to completely inhibit the production was found to be the presence of 0.5 mg phytic acid /100 ml medium.

It is evident from this experiment that as the amount of phytic acid is increased, the production of AFB_1 is gradually decreased and completely inhibited by an amount > 0.5 mg/100 ml. It has been reported previously that metal ions are important for optimal growth of fungi and a chelator may affect the production of AFB_1 by altering the growth conditions of the fungi (Ehrlich and Ciegler, 1985; Lee *et al.*, 1966; Lillehoj *et al.*, 1974; Reddy *et al.*, 1971).

Synthetic liquid mediums have been used extensively to study the growth and production of toxins and/or the effect of added nutrients in the cultures (Dayi *et al.*, 1995; Ehrlich and Ciegler, 1985; Lee *et al.*, 1966; Lillehoj *et al.*, 1974; Reddy *et al.*, 1971). They provide a constant amount of nutrition to all cultures and growth environment to the mycelium and have been helpful to optimize the production patterns of aflatoxins also. Reddy *et al.* (1971) studied different media for the biosynthesis of aflatoxin and reported the requirement of potassium and magnesium for aflatoxin production. Several studies were conducted to find the cultural conditions that affect toxin production. It had been reported that besides the requirement of other physical factors (temperature and pH) (Lee *et al.*, 1966) growth factors, i. e., Fe, Cu, Cd, Zn, Mg and K ions, are also required for aflatoxin biosynthesis in different corn substrates (Lillehoj *et al.*, 1974). Among these studies, the effect of trace elements on aflatoxin

production is most interesting. It has been suggested that metal chelates are formed by the trace elements, especially zinc and ferrous, and that they function in the activation of metalloenzymes or work as an enzyme activator (Dwyer and Mellor, 1964) required during the process of aflatoxin synthesis. Since phytic acid has been shown extensively to be a strong chelator *in vivo* and *in vitro* studies, it is not surprising to see the inhibition of aflatoxin production from *A. flavus* in the presence of phytic acid.

The first trial was conducted to find the optimum dose of phytic acid to inhibit the process of AFB_1 synthesis by *A. flavus*. To confirm whether metal ions play any role in the biosynthesis process of aflatoxins, a second experiment was conducted to study the effects of different metal ions. This trial explored the possible role of metal ions in combination with phytic acid as growth factors for aflatoxin biosynthesis.

2. Effect of Metal Ions and Phytic Acid on the Production of Aflatoxin in Liquid Medium

The results of the previous trial showed the biosynthesis of AFB_1 in the Czapek-Dox liquid medium inoculated with *A. flavus*. It was observed that although treatment containing 0.1mg phytic acid reduced AFB_1 biosynthesis, the concentration of phytic acid in the liquid medium however, was not sufficient to completely inhibit the AFB_1 biosynthesis. This current study was conducted to evaluate the potential role of metal ions in the AFB_1 biosynthesis at the same concentration of phytic acid (0.1mg/100ml). The objective of this study was to observe whether this amount of phytic acid could chelate the metal ions from

other sources in the medium and to explore whether the absence of metal ions affects aflatoxin biosynthesis. It was assumed that potentially there would be other sources of metal ions in the medium, i. e., water, added corn, reagents, so it is likely that *A. flavus* would be able to grow without metal ions or that lower amounts of phytic acid could chelate those sources as well. The results of this study are presented in Appendix B.

The results of this study (Figure 4.3) indicate that, in the absence of $FeSO_4$ and $ZnSO_4$ (treatment E and G, respectively) the production of AFB_1 was completely inhibited and the production of aflatoxin was significantly ($p<0.05$) different from the control treatment A. However, the absence of $CuSO_4$ and $MgSO_4$ (treatment D and F) did not inhibit the AFB_1 production completely, but the synthesis of aflatoxin was significantly lower than that of the control (treatment A) in the absence of $MgSO_4$ (treatment D), whereas the absence of $CuSO_4$ (treatment F) did not significantly reduce the AFB_1 biosynthesis (Figure 4.3).

Previously, it was reported that different growth and cultural conditions affected the production of aflatoxins in submerged culture. The effect of trace elements of aflatoxin production was most interesting, especially the zinc requirement. It has been suggested that metal chelates are formed by the trace metals and that they function in metalloenzymes or as enzymatic activators (Dwyer and Mellor, 1964), and that the stability of the metal complexes may depend on the pH of the medium in which they are formed (Lee *et al.*, 1966).

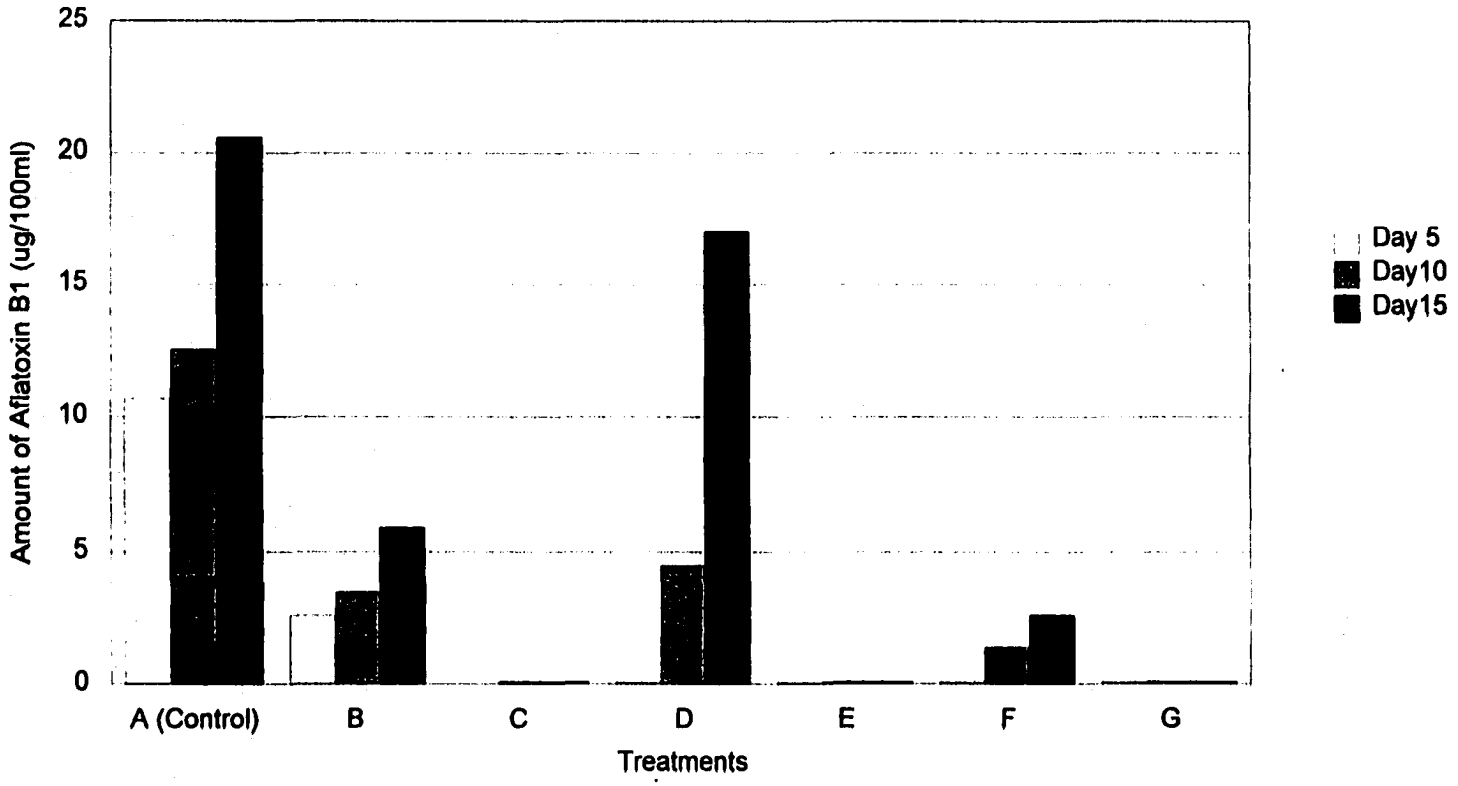

Figure 4.3 Effect of metal ions on the production of AFB_1 from *A. flavus* in Czapek-Dox liquid medium. Values are mean +/- standard error of three replications. (A=Control; B=0.1mg phytic acid/100ml; C=1mg phytic acid/100ml; D=0.1mg phytic acid/100ml-$CuSO_4$; E=0.1mg phytic acid/100ml-$FeSO_4$; F=0.1mg phytic acid/100ml-$MgSO_4$ G=0.1mg phytic acid/100ml-Zn SO_4)

Since the presence of metal ions is required by *Aspergillus* for the synthesis of aflatoxins, the hypothesis of this study was based on the chelating properties of phytic acid towards the inhibition of toxin production. It has previously been reported that during the growth process of fungi, respiratory activity of fungi is reduced, resulting in the accumulation of acetyl coenzyme A (Ach-A) and other intermediates of the tricarboxylic cycle (Detroy, 1970; Dayi *et al.*, 1995). As a result of this inductive effect of intermediates, the secondary biological synthesis system is activated leading to the commencement of AFB_1 synthesis by fungi (Dayi *et al.*, 1995), and this secondary synthesis is regulated by the metal ion dependent or metalloenzymatic pathway. When small amounts of the chelators, i. e., phytic acid, are added to the culture, metal ions become unavailable for metabolic activation such as the process of enzymatic activation, thus resulting in the inhibition of AFB_1 biosynthesis by *A. flavus*.

Phytic acid is found in most cereals and has been implicated as anti-nutritive because of its chelating properties with the divalent ions in foods. It has been reported previously that trace elements in most cereals occur in the aleurone layer (Lee *et al.*, 1966; Lillihoj *et al.*, 1974) and support the growth of *Aspergillus* (Marsh, 1975) with the exception of corn where they occur predominantly in the germ portion (Burow *et al.*, 1997; Zhou and Erdman, 1995). The results of this study also suggest that when seeds of plants are sprayed or soaked with phytic acid solution with appropriate concentration prior to storage, protection from mold would be achieved. Furthermore, their

treatment could also reduce the incidence of cancer by inhibiting the biosynthesis of aflatoxins.

The next phase of this study is designed to evaluate the effects of these factors/recommendations in clean corn samples on aflatoxin biosynthesis, where indigenous activity of phytic acid and other components may have the same effect.

5. EFFICACY OF INTRINSIC PHYTIC ACID AND LINOLEIC ACID ON THE PRODUCTION OF AFLATOXINS BY *ASPERGILLUS FLAVUS* (LINK EX. FRIES) IN CORN STORAGE STUDY

A. Introduction

Corn (*Zea mays*) is an important crop in the grain and livestock economy worldwide. Grain colonization by *Aspergillus* spp. and subsequent biosynthesis of aflatoxins are chronic problems in agriculture. In the beginning, the production of aflatoxin was thought to be a post-harvest problem due to inadequate storage conditions until a more severe problem in the pre-harvest crops in the field was found. Aspergilli typically gain access to the seed through cracks generated by environmental stress and/or *via* insect damage. Aflatoxins are naturally produced as the secondary metabolism of fungi and their presence in the feed and food crops cannot be completely avoided. Aflatoxin B_1 (AFB_1), the most potent of mycotoxins, causes primary liver cancer (PLC) through necrosis, immune-suppression, gastrointestinal tract dysfunction, and pulmonary edema, in animals and humans. It is metabolized by the phase I enzyme system resulting in the production of highly reactive epoxides, which cause damage to cells by co-valently binding to the proteins and DNA.

Several methodologies, based on physical, chemical and biological principles, have been developed for the decontamination of aflatoxin affected crops. Although chemical treatments have provided the most relief through their direct and indirect interaction with either mold or aflatoxins, the decontamination products of such treatments are however, still under intensive

investigations. The most thorough investigations of seed pathogenesis have been conducted with maize kernels, in which *Aspergillus* spp. preferentially colonizes the lipid-rich embryo and aleurone tissues (Burow *et al.*, 1997). During these investigations, other factors, i.e., protein inhibitory compounds (Huang *et al.*, 1997), linoleic acid (Burgos-Hernandez, 1998), phytates (Hamid and Smith, 1987) and lipoxygenase by-products (Zeringue *et al.*, 1996) have been shown to reduce the mutagenicity of aflatoxins.

The role of CP-450s on AFB_1 formation, degradation, and its epoxidation pathways has been extensively reported in different animal models and human liver (Pelkonen *et al.*, 1997; Roy and Kulkarni, 1997; Guengerich *et al.*, 1998). Several reports have reported that the degradation of aflatoxins takes place due to the involvement of fungal cytochrome P-450 (CP-450) monoxygenase enzyme systems in the degradation of aflatoxin B_1 and G_1 by intact mycelium and cell-free extracts of *A. flavus* (Hamid and Smith, 1987).

Compounds consumed in our diet may also inhibit the enzymatic activity. Extensive research in the natural substances of plants has identified innumerable natural anti-mutagenic components which either activate the phase II detoxification enzymes or inactivate the phase I enzyme system, required for several indirect-acting mutagens to become mutagens. Currently, the role of plant derived dietary fibers in the protection of human mutagenesis and carcinogenesis has been extensively studied. The major dietary fibers come from cereal grain or bran-milling fraction. Numerous antioxidative compounds have been identified in the fibrous portion of these cereals such as

alkylresocinol, saturated and unsaturated lipids etc. Graf and Eaton (1985) have recently identified an additional component besides fiber, the phytic acid. Substantial evidence in animal studies have proved that phytic acid (inositol hexaphosphate), a component of the dietary fiber complex, reduces the risk of large intestinal cancer (LIC). It has been assumed that phytic acid, an intrinsic component of grains, can render protection against cancer through its antioxidant property (Shamsuddin, 1995). The ability of phytic acid to bind with metals especially iron (Fe^{2+}) has been attributed to its antioxidant and anticarcinogenic activity. By chelating Fe^{2+}, phytic acid inhibits Fe-induced free radical (.OH) generation (Graf and Eaton, 1990). Subsequently, it limits the processes of lipid peroxidation and DNA damage by inhibiting free radicals formation, which are thought to be involved in the etiology of certain cancers. However its role in the soybean has remained controversial due to its anti-nutritive chelating properties and on the other hand its beneficial inhibition of *Aspergillus* spp. invasion (Hamid and Smith, 1987)

The actual mode of action of phytic acid is not known except for its chelation and antioxidant properties in the meal matrices, but it is assumed that it might have the capacity to bind to the toxin(s) and/or to produce some less reactive metabolites through its binding ability to the hydroxylated form of aflatoxin. Phytic acid might also inhibit the formation of toxins by the respective molds through its antioxidant properties.

The objective of this study was to evaluate the role of phytic acid and its other counterparts, i.e., linoleic acid, towards the production of aflatoxins during

corn storage. It was also hypothesized that the presence of phytic acid along with other antimutagenic factors in corn such as linoleic acid, which is part of the initial substrate for phytic acid formation, the phosphotidylinositol, would affect the formation and/or toxic/mutagenic potential of aflatoxins.

B. Materials and Methods

1. Corn and Fungal Cultures

Non-contaminated whole kernel corn samples were obtained from Cargill (Port Allen, Baton Rouge-Louisiana). *Aspergillus flavus* (Link ex. Fries) was kindly provided by Dr. Kanneth Damann (Department of Plant Pathology, Louisiana State University, Baton Rouge, LA).

2. Chemicals

Trifluoroacetic acid was purchased from Sigma Chemical Co. (St. Louis, MI). Methanol (HPLC grade), citric acid monohydrate, dimethyl sulfoxide (DMSO), ethyl ether anhydrous, hexane (HPLC grade), potassium chloride, potassium phosphate dibasic anhydrous, and water (HPLC grade) were acquired from Mallinckrodt Baker Inc. (Paris, KT). Acetonitrile (HPLC grade) was purchased from Baxter Co. (Muskegon, MI). Ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KT). Potato dextrose agar and crystal violet were ordered from Difco Laboratories (Detroit, MI). Multifunctional cleanup system (MFC) (Mycosep Romer column # 224) was obtained from Romer Laboratories Inc. (Washington, MO).

3. Fungal Growth and Harvesting Culture Inoculum

Fungi (*A. flavus*) was cultured on potato dextrose agar (PDA). In order to prepare the broth medium, 39 g of potato dextrose agar was dissolved in 1 L of sterile distilled water and was autoclaved at 121° C for 30 min. After the broth temperature reached about 45° C, the medium was aseptically poured on the sterile petri dishes (15 x 25 mm). The plates were inverted after the agar was hardened. The plates were seeded with *Aspergillus flavus* (AF-13 Link ex. Fries) plugs under the laminar hood and were inoculated in an incubator for 7 days at 30° C. Cultured material was scraped off and rinsed with sterile distilled water (containing 0.1% Tween-20) (ca. 10 ml/plate). The mold/water suspension from each plate was collected to make a stock inoculating conidial suspension. The concentration of stock inoculating conidial solution was calculated by using a hemacytometer/microscope. For the experiment, a conidial suspension of 10^7 conidia/ml was prepared by diluting the stock conidial solution in sterile distilled water.

4. Corn Inoculation

Inoculation of the corn culture was performed according to the method reported by Leslie *et al.* (1992) with modifications. Briefly, yellow, aflatoxin free whole corn kernels were coarsely cracked in the Willey Mill, then homogenized in a tumble blender (ground corn storage study). For the whole corn treatment, healthy intact seeds were separated manually (whole corn

storage study). Experimental protocol for each treatment is depicted in Tables 5.1-5.2.

Table 5.1 Corn *Aspergillus flavus* inoculation design for phytic acid treatment (whole corn kernels)

Sample	N	Heat Treatment	Water Added	Inoculum/Tmt
A (-control)	15	121° C; 30 min.	24 ml	No inoculation
B (+control)	15	121° C; 30 min.	24 ml	Inoculation
C*	15	121° C; 30 min.	24 ml	Inoculation/Tmt.
D**	15	121° C; 30 min.	24 ml	Inoculation/Tmt.
E***	15	121° C; 30 min.	24 ml	Inocula/Tmt.

* C = Phytic Acid (1g/50g corn) in Water
** D = Linoleic Acid (1g or 1ml/50g corn) in Hexane
*** E = 0.5 g Phytic Acid + 0.5ml Linoleic Acid /50g corn

Briefly, samples (50 g) were weighed individually into sterile 100 ml beakers, and 10 ml of sterile distilled water was added to each beaker for both whole corn (A-E treatments) and ground corn (F-J treatments) samples. The beakers were covered with a cheesecloth plug and aluminum foil; then autoclaved at 121° C for 30 min. After autoclaving, the ground corn and whole corn kernels were aseptically poured in petri dishes (25 x 150 mm) under the laminar hood. Another 12 ml of water (in case of treatments A, B, F and G only), and/or phytic acid solution in 12 ml water was aseptically added to each of the plates (in the case of treatments C and H only). One ml of the conidial suspension (ca. 10^7 conidia/plate) was added to their respective plates by either combining with a second batch of 12 ml water (treatment B only) or along with

10% phytic acid solution (treatment C and H containing 1g phytic acid/50g corn). For the linoleic acid treatment (treatments D and I), linoleic acid (1g/50g corn) in n-hexane was added. Similarly in the linoleic/phytic acid combination treatment (treatments E and J only), 0.5 g linoleic acid in n-hexane, and 0.5 g phytic acid in water/50 g corn were added to each plate aseptically. No *A. flavus* inoculum was added to treatments A and F.

Table 5.2 Corn *Aspergillus flavus* inoculation design for phytic acid treatment (ground corn)

Sample	N	Heat Treatment	Water Added	Inoculum/Tmt
F (control)	15	121° C; 30 min.	24 ml	No inoculation
G (control)	15	121° C; 30 min.	24 ml	Inoculation
H*	15	121° C; 30 min.	24 ml	Inoculation/Tmt.
I **	15	121° C; 30 min.	24 ml	Inoculation/Tmt.
J***	15	121° C; 30 min.	24 ml	Inoculation/Tmt.

* H = Phytic Acid (1g/50ml corn) in Water
** I = Linoleic Acid (1 g or 1ml/50g corn) in hexane
*** J = 0.5 g Phytic Acid + 0.5 ml Linoleic Acid /50 g corn

The plates were incubated in an incubator (VWR Scientific, model 2015) for 35 days at 30° C. Samples (3 plates/treatment) were removed from the incubator at 0, 4, 7, 14, 21, and 28 days of incubation.

The samples were transferred and placed in sealed plastic bags and stored at -20° C until extraction and for the high performance liquid chromatography (HPLC) analysis for aflatoxin determination.

5. Safety Precautions

Since a highly toxigenic strain of *Aspergillus* was used in this study, several safety measures were required to avoid exposure to toxic material and mold spores. A half-face mask respirator equipped with HEPA filters was used every time the corn samples were handled. Also, protective eye wear and nitrile gloves were worn at all times. All samples were handled in a biological laminar flow hood. During the last portion of the inoculation study and the toxin extraction, high sporulation required maximum safety precautions due to highly volatile spores. Each and every area including the hood was thoroughly sterilized with 95% ethanol.

6. Toxin Extraction

Whole corn and ground corn samples were extracted for aflatoxins following the method reported by Wilson and Romer (1991). Corn samples (50 grams) were placed in a blender with 100 ml acetonitrile:water (9:1) and blended at high speed for 2 minutes. The extract slurry was filtered through Whatman # 4 filter paper. The filtrate was considered the crude corn extract.

7. Aflatoxin Purification

The samples were analyzed following the method reported by Wilson and Romer (1991) with slight modifications. All the crude corn extract samples from day 0 through day 35, already in acetonitrile:water (9:1) were purified through the Multifunctional cleanup system (MFC) (Mycosep Romer column # 224, Romer Laboratories Inc. Washington, MO). Briefly, 2 ml of crude corn extract were placed in the culture tube and the flanged-end of the column were

pushed into the extract, letting the crude extract pass through the column. Approximately, 1 ml of the purified extract was collected in amber vials and stored at 4° C until an HPLC analysis (Figure 5.1).

8. Quantification of Aflatoxins by High Performance Liquid Chromatography (HPLC)

The samples were analyzed following the method reported by Wilson and Romer (1991). All the samples from day 0 through day 28, already purified through Mycosep Romer column # 224 were used to determine the amount of aflatoxins.

To derivatize the aflatoxins, a 200 µl aliquot of the purified extracts was transferred into an HPLC auto-injector vial (Waters, Milford, MA), and 700 µl of trifluoroacetic acid derivatizing reagent [distilled water: trifluoroacetic acid: acetic acid (7:2:1)] were added. The samples were placed in a waterbath at 65° C for 8.5 minutes. The vials were cooled in an ice-water bath.

The samples were placed in an HPLC auto-sampler (Waters 717plus auto-sampler, Waters, Milford, MA). The analysis was carried out using a Waters modular system with a Waters 470 scanning fluorescence detector (360 nm excitation and 440 nm emission) and a Novapak C_{18} reverse phase column. Fifty µl of sample were injected and analyzed using a water:acetonitrile (4:1) mobile phase with a flow rate of 2.0 ml/minute. Approximate retention time for aflatoxin G_1 (G_{2a}), B_1 (B_{2a}), G_2 and B_2 were 2.6, 3.5, 7, and 9.8 minutes, respectively. Aflatoxin concentrations were calculated by using the Millenium Chromatography Manager Software (Waters Inc., Milford, MA).

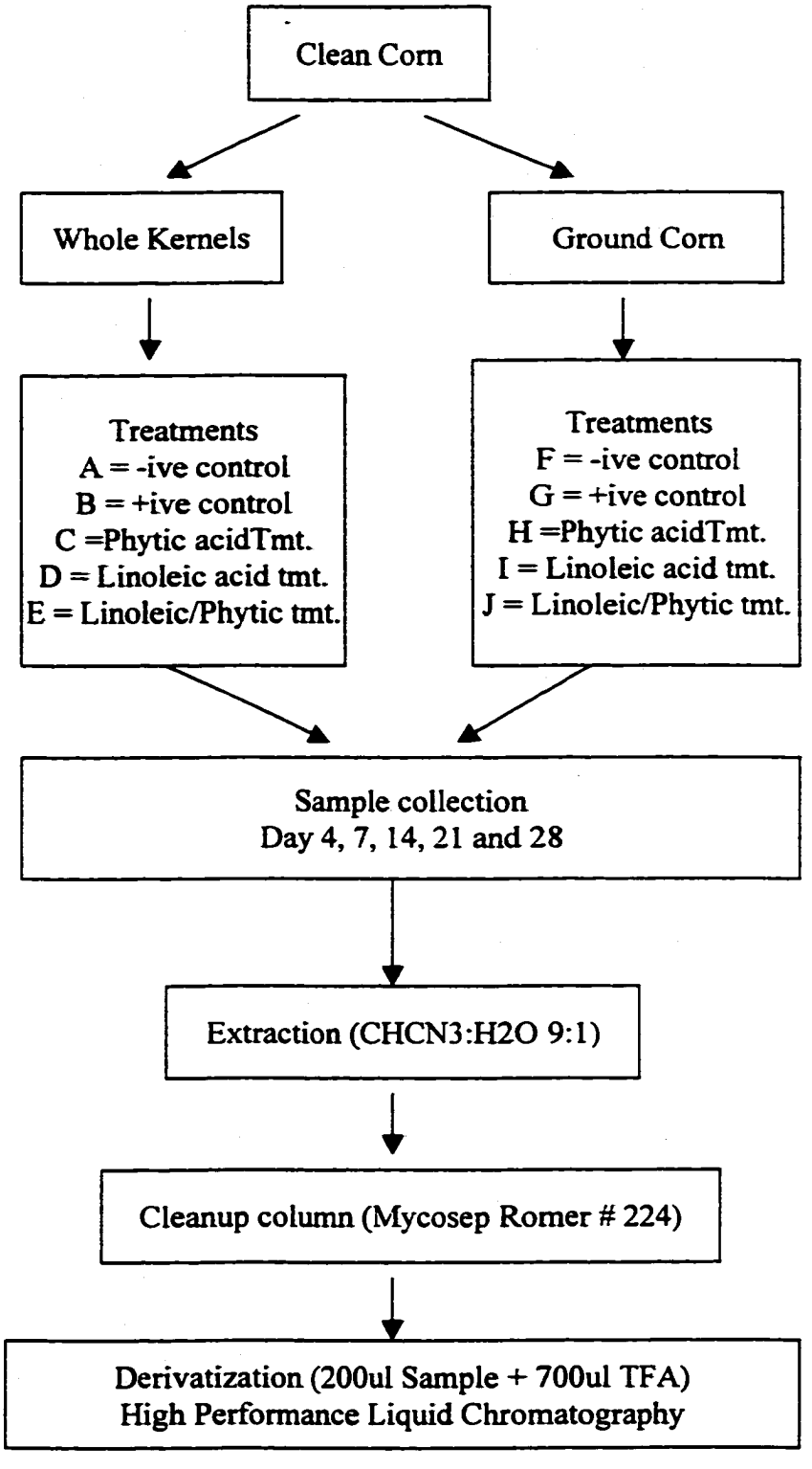

Figure 5.1 Flow diagram for the corn storage study. Values are mean +/- standard error of three replications.

9. Statistical Analysis

Statistical analysis of the data was performed by using the SAS systems software (SAS, 1988). One- and two-way analysis of variance (ANOVA) was used to determine the differences between treatments and toxin production. Scheffe's test with an alpha value of 0.05 ($P<0.05$) was used to compare differences among the treatments.

C. Results and Discussion

1. *Aspergillus* Growth and Effect on Toxin Production

The growth of *A. flavus* was visually observed during four weeks of study to determine the onset of growth and to estimate the extent of growth and sporulation. According to visual observations, a gradual to no growth of fungi was observed in all treatment samples during the first week of study except in *Aspergillus*-positive controls (treatment B & G), where a fast sporulation was evident from the second day of study. No growth was observed in most of the treatments and *Aspergillus*-negative controls in the first few days of observations. As was expected, copious growth was observed within a few days in the *Aspergillus*-positive control, however, the treatments (especially phytic acid) and aspergillus-negative control samples provided a constant resistence to the mold growth. During the second week the sporulation showed an incremental invasion of the culture. After the first two weeks, *A. flavus* had invaded the whole media at a faster rate irrespective of treatments or controls.

These observations are consistent with the previous studies (Doyle and Marth, 1978; Hamid and Smith, 1987; Karuaratne and Bullerman, 1990; Lopez-Garcia, 1998). Production of toxin was found directly proportional to the fungal sporulation (which was observed visually) in the aspergillus positive controls in both whole kernels and ground corn (Figure 5.2) compared to treatments and aspergillus-negative controls. It is interesting to note that production of aflatoxins was faster in the whole kernels (treatment B) than in the ground corn of *Aspergillus*-positive controls (treatment G). This phenomenon can be explained by the fact that ground corn would result in the exposure of intrinsic antitoxin/antifungal components, i. e., protein inhibitory compounds (Huang et al., 1997), enzyme systems (Applebaum,1980), lipids and lipoxygenase related metabolites (Zeringue, 1997), and/or phytates, to the mold, which can be inhibited and/or repress the growth of the fungi and reduce toxin production, subsequently.

The production of aflatoxins in ground corn showed a linear increment during the first two weeks of sporulation but started to decline afterwards as evident also in whole kernels after three weeks. On the contrary production of aflatoxins in *Aspergillus*-negative controls showed a linear increase in the whole kernels whereaas ground corn did not (Figure 5.3). Aflatoxin production was found independent of mode of interaction, whether naturally contaminated or inoculated with *Aspergillus flavus*. This phenomenon is consistent with previous studies (Hamid and Smith, 1987; Doyle and Marth, 1978a-j; Doyle and Marth, 1979; Guo *et al.*, 1995; Huynh and Lloyd, 1984; Lopez-Garcia, 1998).

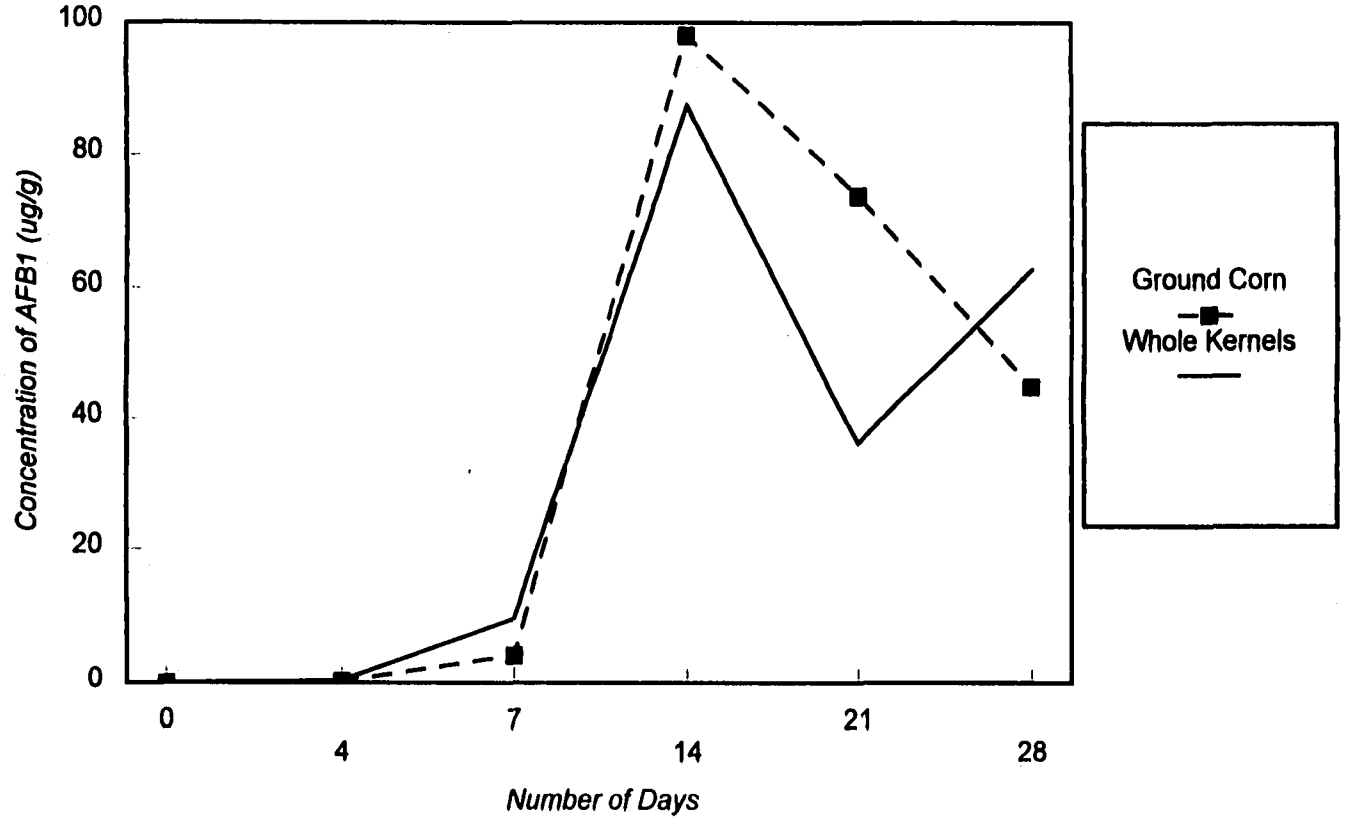

Figure 5.2 Production of aflatoxin B_1 in whole kernels and ground corn without *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

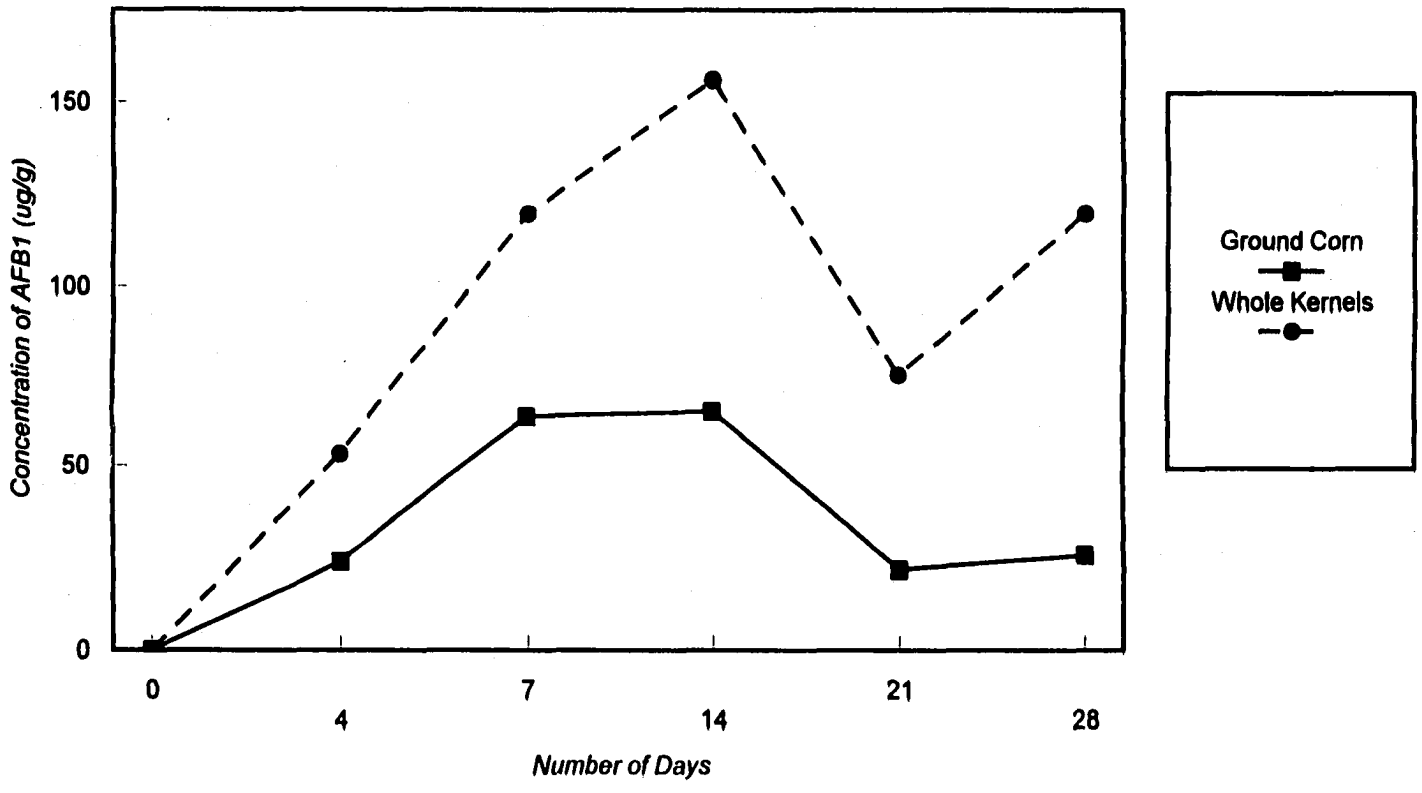

Figure 5.3 Production of aflatoxin B_1 in whole kernels and ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

Figures 5.4 and 5.5 depict the production of AFB_2. The production of AFB_2 was found significantly higher in aspergillus-positive whole kernels than aspergillus-positive ground corn at day 14 and day 28 samples (Figure 5.5). The pattern of toxin production was found somewhat similar to AFB_1 production.

During the next two weeks of study, an interesting pattern of aflatoxin production emerged, which was reminiscent of the *Aspergillus*-negative control. The production of AFB_1 reached a climax by the 14th day of study as observed in the growth of fungi, which had invaded the plates in a similar fashion during the first two weeks of visual observations. This phenomenon is also in close agreement with previous studies (Doyle and Marth,1978a; ; Doyle and Marth, 1978b; Doyle and Marth, 1978c; Doyle and Marth, 1978d; Doyle and Marth, 1979; Guo *et al.*, 1995; Ciegler *et al.*, 1966). These studies explained this trend was dependent on the provision of the nutrition to the mold to support their growth. Furthermore, a more elaborate picture of the set of events in which the fungi, after exhausting all the available substrate, starts to depend their own by-products, mycelium and aflatoxins, can judiciously explain this phenomenon. Hamid and Smith, (1987) reported that molds that produce aflatoxin could degrade them too. In an attempt to elucidate the conditions in which toxigenic *Aspergillus* spp. degrade the same aflatoxins they produce, Doyle and Marth studied different sets of the conditions in a series of experiments that govern degradation of aflatoxin by these molds (Doyle and Marth, 1978a; Doyle and Marth, 1978b; Doyle and Marth, 1978c; Doyle and Marth, 1978d; Doyle and Marth, 1979). They reported that the ability of *Aspergilli* to degrade aflatoxin

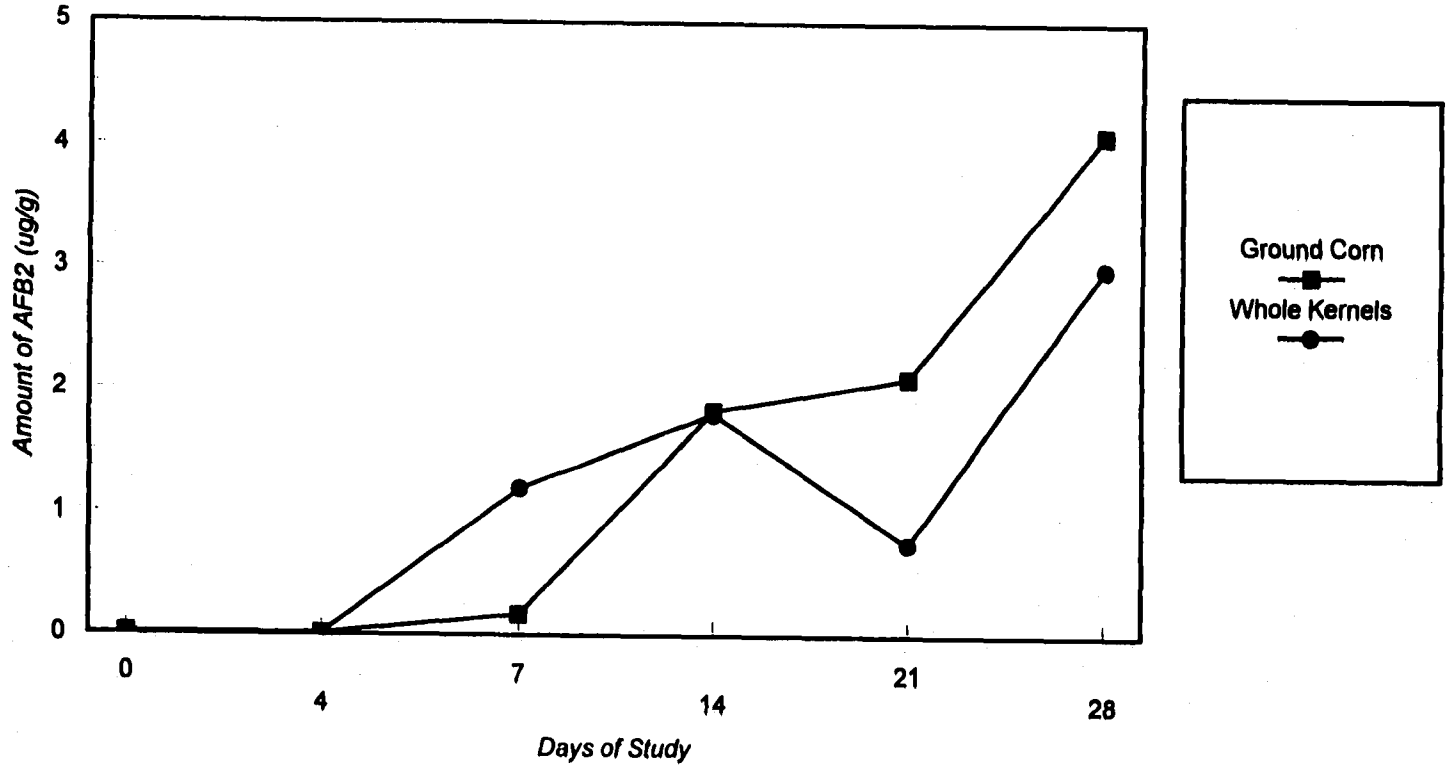

Figure 5.4 Production of aflatoxin B_2 in whole kernels and ground corn without *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

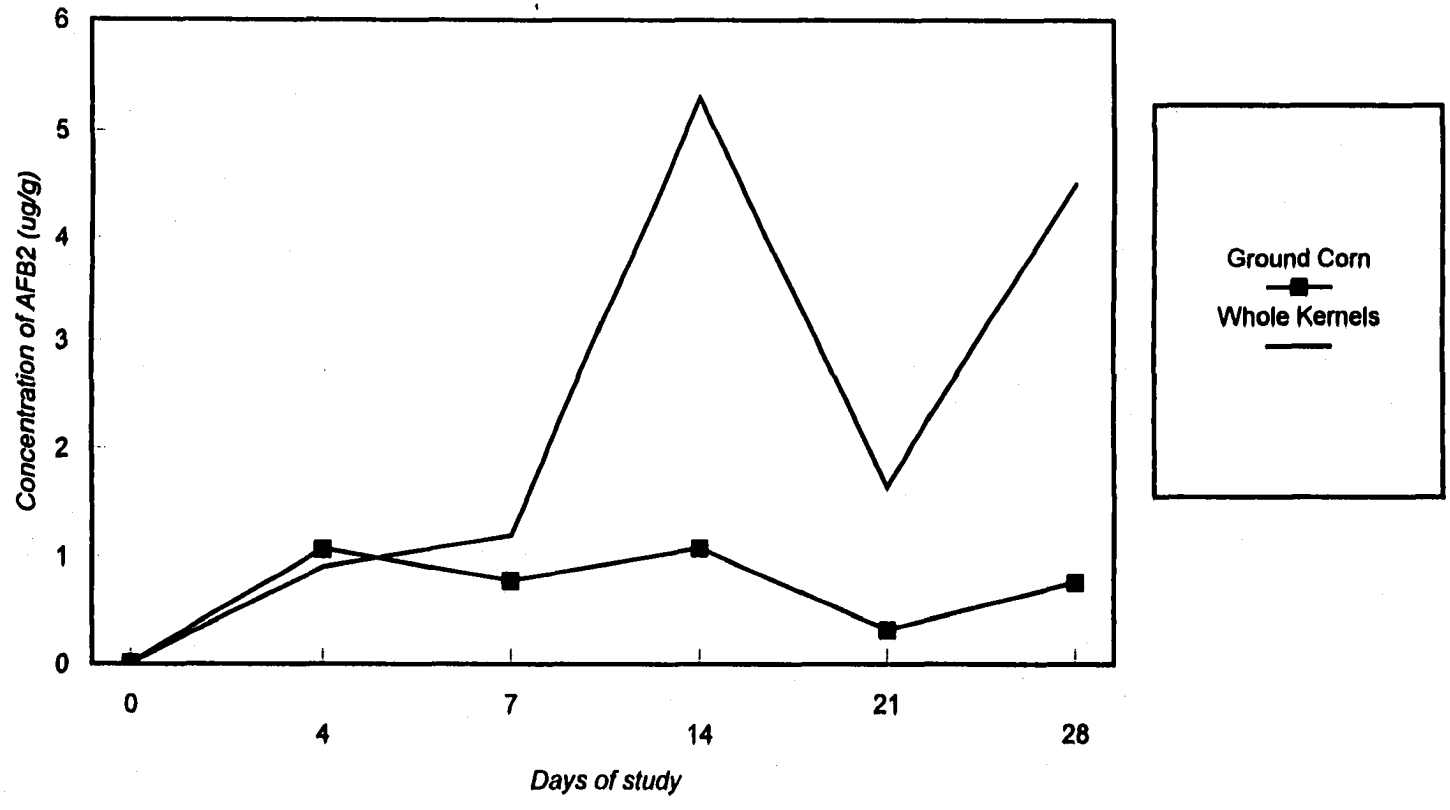

Figure 5.5 Production of aflatoxin B_2 in whole kernels and ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

was dependent on: (a) the age of mycelia: 8 to 10-day-old mycelia were generally most effective in degrading aflatoxin B_1 (Doyle and Marth, 1978); (b) disruption of mycelia: blended or fragmented mycelia actively degraded aflatoxin while intact mycelia did not (Doyle and Marth, 1978a; (c) the substrate used to produced the mycelia – substrates that support substantial growth of mycelia yield mycelia having the greatest ability to degrade aflatoxin (Doyle and Marth, 1978b); (d) the strain of *A. parasiticus* or *A. flavus* – strains that produced larger amounts of aflatoxin generally degraded more aflatoxin; (e) the amount of mycelia in the reaction mixture – the rate of degradation increased as the amount of mycelia was increased (Doyle and Smith, 1978d); (f) the amount of aflatoxin in the reaction mixture – the rate of degradation increased as the amount of aflatoxin was increased; (g) temperature – maximum activity occurred at 28 °C; and (h) pH—maximum activity occurred at pH 5-6.5 (Doyle and Marth, 1978).

In another study Huynh and Lloyd (1984), studied the age of mycelium in the production of aflatoxins. They observed that the age of mycelia is critical for the growth of fungi and production of the secondary metabolites. They compared three different isolates of *A. parasiticus* according to their age. Maximum yield was achieved in the younger mycelium within 14 days of growth and afterwards a decline in the amount of aflatoxin production was observed as the mycelial culture aged. Young mycelia (4days old) synthesized the greatest amount of toxin, but the aging mycelia (14 days old) were mainly

responsible for degradation. The same phenomenon was observed in our *Aspergillus*-positive and -negative controls. As the mycelia aged, the production of aflatoxin was reduced over a period of time.

2. Toxin Production and Treatments

a. Effect of phytic acid on aflatoxin production

HPLC analysis of phytic acid treated corn samples showed that regardless of substrate type (both whole corn and ground corn), *A. flavus* did not produce a significant amount of aflatoxins during the first four days of study (Appendix C), however, there was a significant difference ($p<0.05$) between *Aspergillus*-positive controls and phytic acid treatments throughout the period of study. Although phytic acid treated whole corn samples produced a higher amount of aflatoxins than ground corn samples throughout the period of study, the difference was not significant. It was observed that naturally contaminated samples (*Aspergillus*-negative controls) had a similar mode of toxin production during the first ten days (Figure 5.6 and 5.7).

Compared to the positive and negative control samples, the production of aflatoxin in phytic acid treated corn remained in the same range or gradually declined after the second week of study. The differences are not very significant among the sets of same treatment samples collected in the proceeding weeks. However, the phytic acid treated corn samples are significantly different from the positive control samples ($p<0.05$).

The production of AFB_2 was also determined during the whole period of study (Appendixes E and F). According to the results, although a linear AFB_2

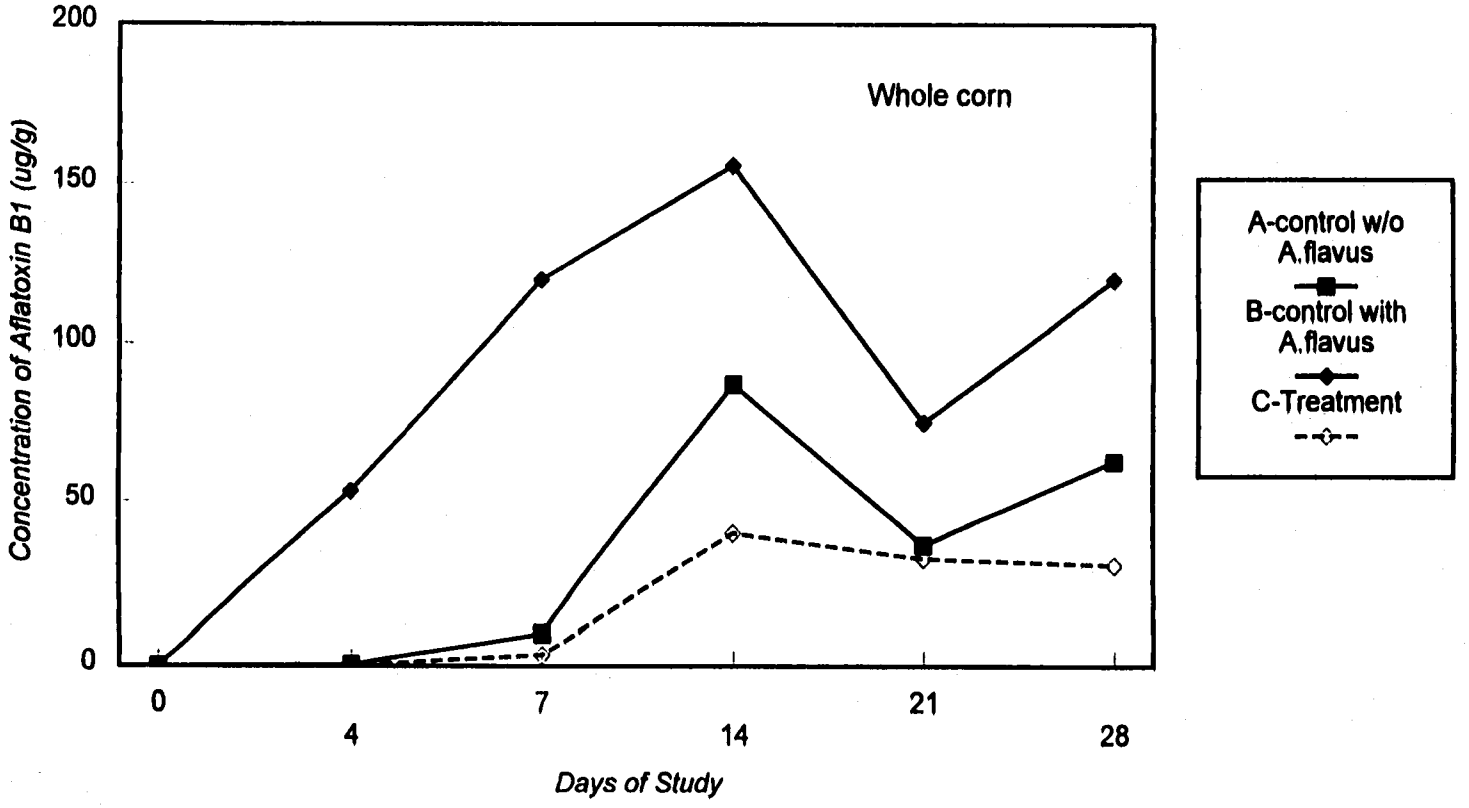

Figure 5.6 Effect of phytic acid on the production of aflatoxin B_1 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

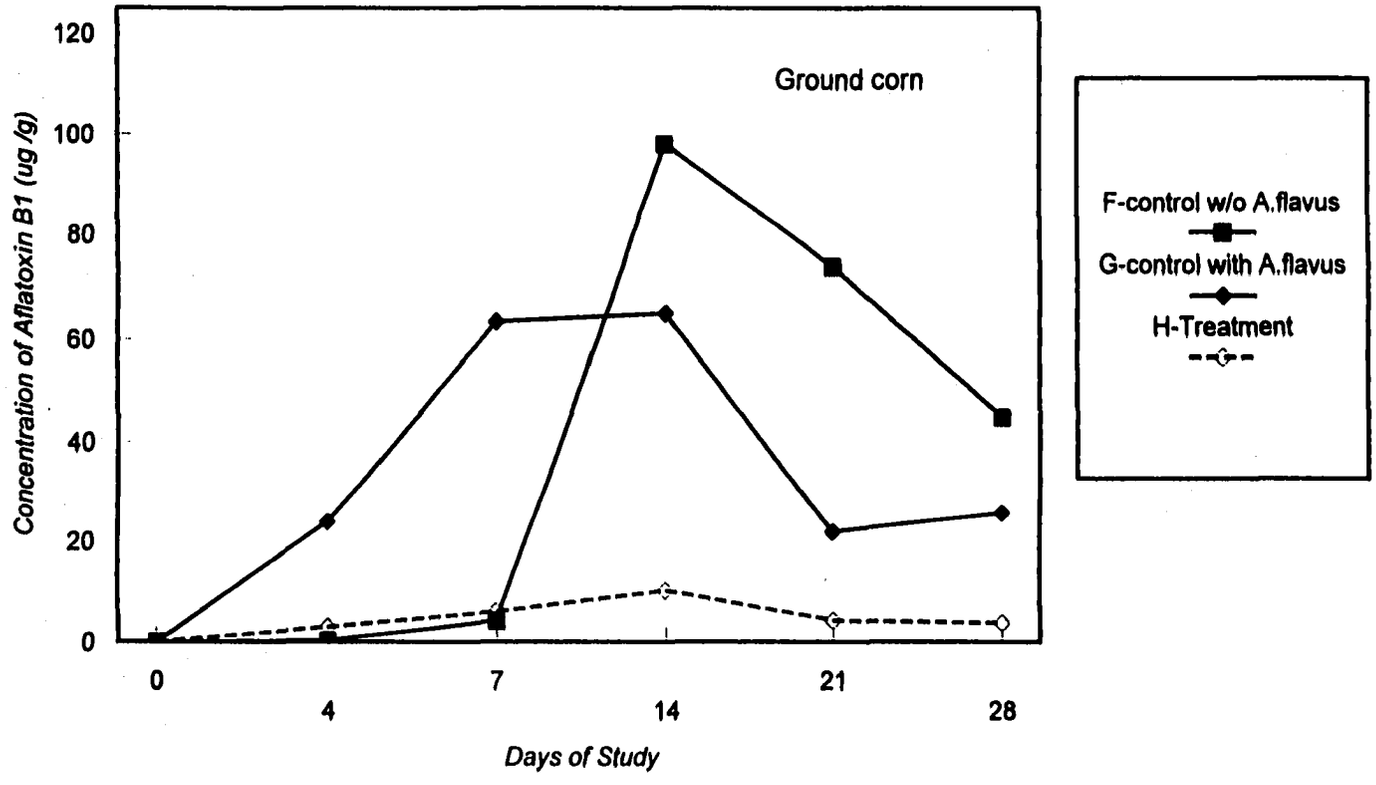

Figure 5.7 Effect of phytic acid on the production of aflatoxin B_1 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

production was noted in both *Aspergillus*-controls, not a very significant amount of AFB_2 toxin was produced in phytic acid treated corn irrespective of substrate type (Figures 5.8 and 5.9). It is interesting to note that there was not a significant difference between aspergillus-negative control and phytic acid treated whole kernels, however, the difference between the aspergillus-positive control and the treatment was significant at day 14 and day 28 samples. In case of ground corn samples, the production pattern was different from the whole kernels. As it is evident that the aspergillus-positive controls and phytic acid-treated samples did not show a significant difference, but there was signifant difference between the aspergillus-negative controls and the treatment at day 14 through the day 28.

Aspergillus flavus is attributed to the production of only B-type metabolites, AFB_1 and AFB_2, whereas *Aspergillus parasiticus* produces both aflatoxin B's and G's metabolites (Pavao, 1995). This study does not provide any evidence of the production of G-type of aflatoxin metabolites.

Phytic acid, the main phosphorus storage of cereals, legumes and oilseeds, is known to bind essential divalent cations, such as calcium, magnesium, manganese, iron and zinc, forming largely insoluble complexes and thereby decreasing their bioavailability. In the previous study inhibition of aflatoxin production was presented by increasing the amount of phytic acid in liquid medium. Corn, the substrate for the production of aflatoxins in this study, is a complex mixture of compounds. It contains a large amount of trace elements in the germ fraction predominantly (Lillihoj *et al.*, 1974). However, phytic acid,

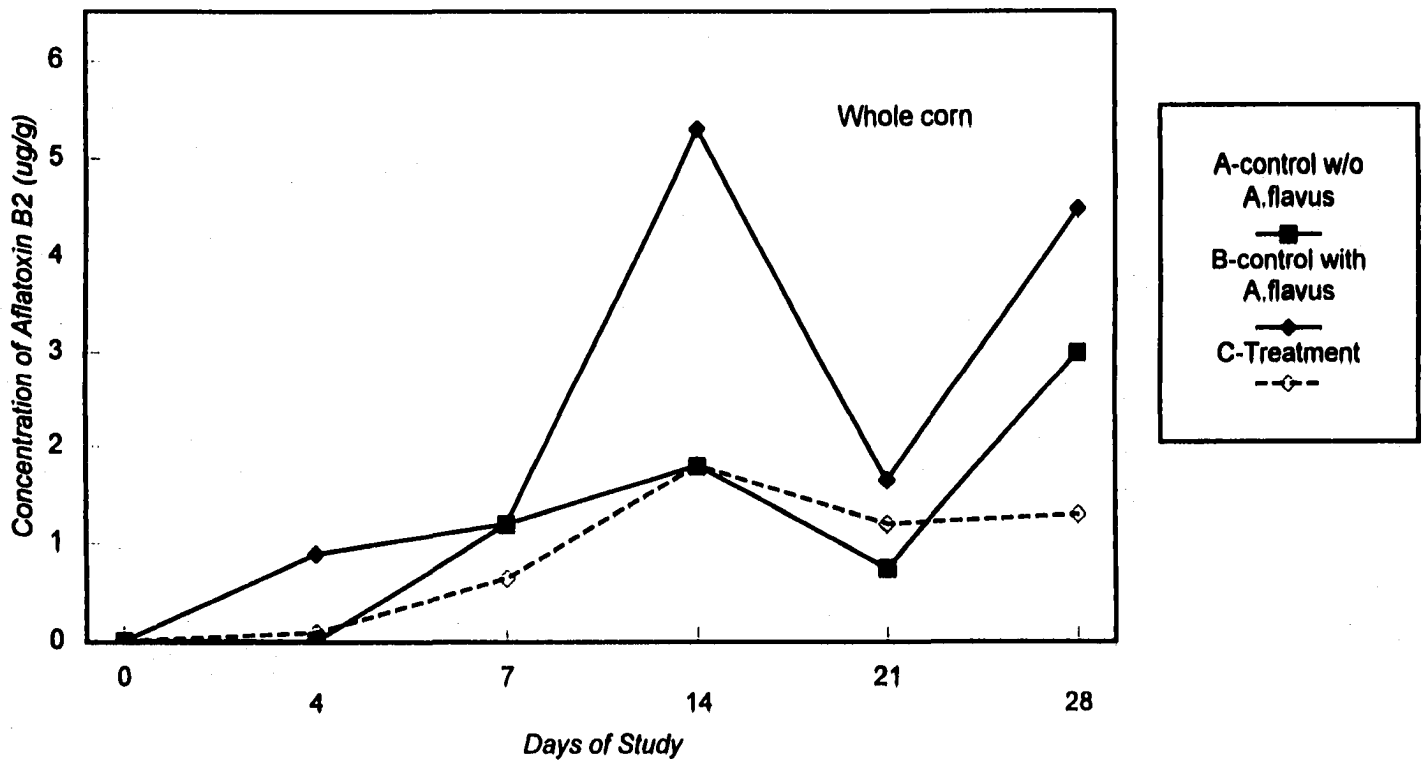

Figure 5.8 Effect of phytic acid on the production of aflatoxin B_2 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

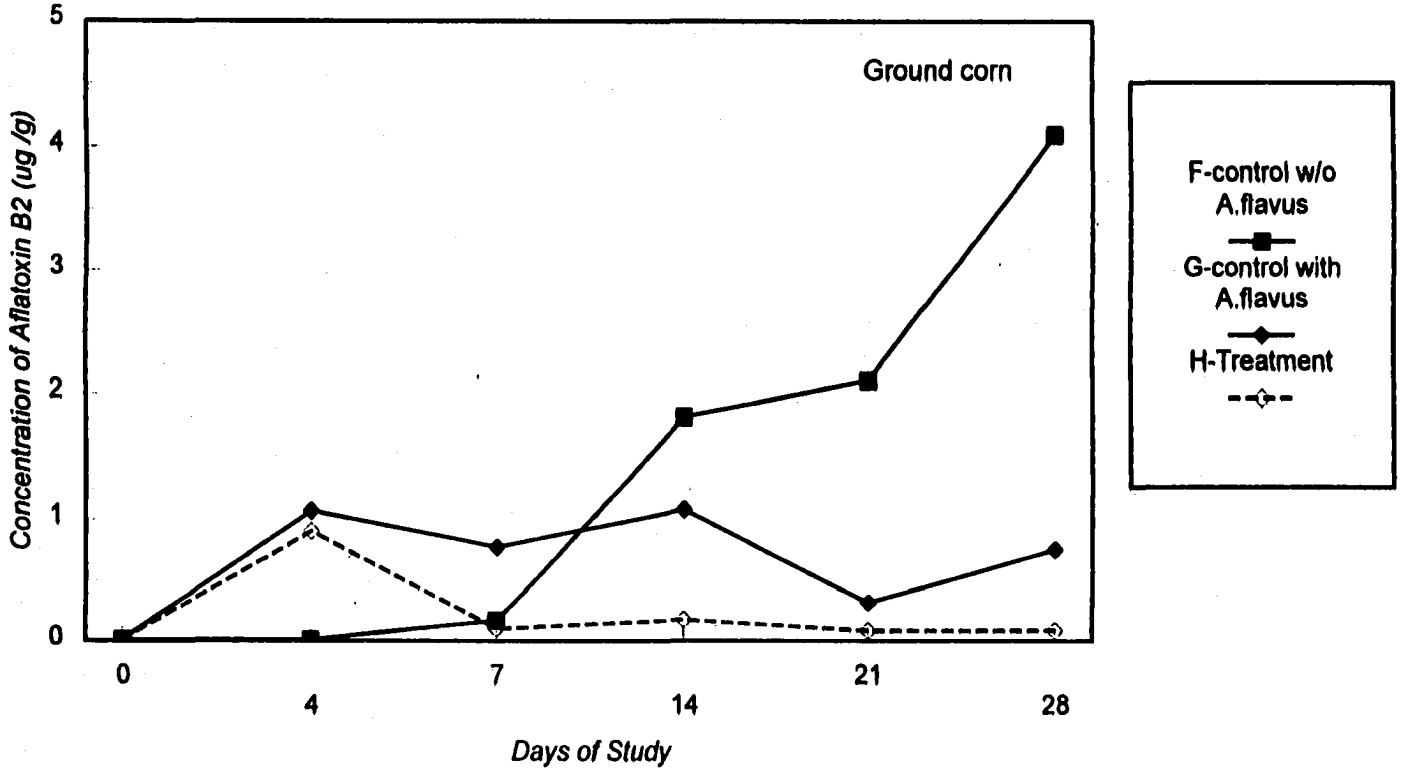

Figure 5.9 Effect of phytic acid on the production of aflatoxin B_2 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

also present in the germ portion, strongly binds several elements, particularly zinc, ferrous, magnesium and copper. It has been reported previously that for promoting the growth of *Aspergillus flavus* and subsequent production of aflatoxins an important role is played by some enzymes which require metal ions as their coenzyme or activator. It is, therefore, unlikely that if the amount of phytic acid or any chelator that could control the activation of those enzymes, is added, can result in the inhibition of the toxin synthesis process. However, a complete inhibition is not evident from the results of this study. According to the results of this study, the production of aflatoxin is significantly lower than that of *Aspergillus*-positive and -negative controls. Since corn is a complex matrix of chemicals other than phytic acid, the possibility of the involvement of other components such as lipids and fatty acids, proteins, and catalytic enzymes, in the biosynthesis process of aflatoxins cannot be completely discounted.

Several studies have reported that soybean is a phytate rich legume and its role in the growth and production of aflatoxins has remained ambiguous. It has been reported that soybeans were a poor substrate for aflatoxin formation as compared to other commodities (Ehrlich and Ciegler, 1985). Gupta and Venkitasubramanian (1975) found that either by autoclaving or by adding zinc to raw unheated soybeans, aflatoxin levels increased more than tenfold. They hypothesized that inhibition of aflatoxin synthesis could be due to the presence of a high amount of phytate, which hindered the toxin formation process by reducing the zinc availability, a necessary micronutrient for aflatoxin biosynthesis.

In a comparative study on *Aspergillus flavus and Aspergillus parasiticus*, it was reported that zinc at relatively high levels inhibited aflatoxin production and that phytate relieved the inhibition (Hensarling *et al.*, 1983) for both *A. flavus and A. parasiticus*. These contradictory results warrants further investigation on the concentration of zinc present in the system. It is not unlikely that if the amount of phytic acid present in the meal matrix is exhausted by the elevated level of metal ions, it can promote the growth and biosynthesis of aflatoxins.

Furthermore, the effect of substrate was also examined in a comparative study with cottonseed, corn and soybean with *A. parasiticus* (Hensarling *et al.*, 1983; Hesseltine *et al.*, 1963). It was observed that reduction of aflatoxin synthesis was found for phytate-added non-sterile corn, but not with the soybean. In cottonseed, phytate added to the autoclaved material caused a significant reduction in aflatoxin production compared to non-autoclaved material. According to Gupta and Venkitasubramanian (1975) phytate is a heat stable compound and the temperature treatment should not have caused any loss of phytate activity. They suggested the role of the enzymes such as phytases in variable amounts in different substrates could have affected the different treatments. In addition to these effects, strong antioxidative activity of phytic acid on lipid peroxidation and on degradation of ascorbic acid has also been reported (Empson *et al.*, 1991). The role of phytic acid in the antioxidation process lies in the obstruction of the oxidative process by chelating/occupying all the available sites of ferrous coordination (Graf and Eaton, 1990).

Phytic acid, by virtue of its chelating properties with metal ions, especially iron and zinc, can be a potent inhibitor of the activity of the fungi to synthesize aflatoxins. Our study was also designed to find the interaction of these metal ions present in corn with intrinsic phytic acid. The results of this study indicate the same pattern of inhibition of aflatoxin synthesis due to the chelation of metal ions by phytic acid. Binding of phytic acid to zinc or iron is believed to result in the low production of aflatoxin compared to aspergillus control treatments; hence, decreased yields of aflatoxin in phytic acid treatments should result from an increased amount of phytic acid. However, the results of this trial do not answer what amount of phytic acid would be required to inhibit the process of aflatoxin biosyntheis.

On the other hand, some studies have shown that phytate is not the only inhibiting source for aflatoxin biosynthesis and besides phytic acid, a role played by lipoxygenases (LOX), present in soy bean, have been elucidated. It was also observed that during the biosynthesis of aflatoxins in soybean and corn, lipoxygenase dependent oxidation of polyunsaturated fatty acids played a major role in the production of aflatoxins (Zeringue *et al.*, 1996). But during this process, some antifungal volatile compounds were also identified (Zeringue *et al.*, 1996, 1997; Croft *et al.*, 1993). The role of soybean isolated hydroxyperoxides, 13S-hydroperoxy-cis-9, trans-11-octadecasdienoic acid (13S-HPODE) and 13S-hydroperoxy-cis-9, trans-11-octadecatrienoic acid (13S-HPOTE), and their aldehyde products were reported to inhibit germination of *A. flavus* spores and/or aflatoxin biosynthesis in maize kernels (Croft *et al.*, 1993).

During this process of oxidation, involvement of phytic acid and its anti-fungal role has not been sufficiently elucidated or has remained controversial.

On the metabolic level, lipoxygenase (LOX) catalyzed pathway plays an important role in the biosynthesis of aflatoxins and some role is played by ferrous ions in the lipoxygenase mediated oxidation process (Zeringue *et al.*, 1996, 1997). Hence, the results of our study also support the above mentioned statement that phytic acid, by chelating the ferrous ions required in the activation of the lipoxygenase enzyme, a precursor for the oxidation process, can inhibit or relieve the formation of aflatoxins. Recently, the role of AFB_1 epoxidation catalyzed by partially purified human liver lipoxygenases has been reported (Roy and Kulkarni, 1997). Apparently, AFB_1 is co-oxidized by lipoxygenases, and by inactivating the lipoxygenases, the pathway of the bioactivation of AFB_1 can be inhibited.

It is interesting to note that different studies depict a controversial picture of the role played by these LOX. The role of LOX inhibitors (Roy and Kulkarni, 1997) and on the other hand the intermediates of lipoxygenase pathway have been reported exclusively (Zeringue *et al.*, 1996; Croft *et al.*, 1993) to inhibit the biosynthesis of aflatoxins from *Aspergillus* spp. The next trial of our study was carried out not only to explore the role of linoleic acid, a major component in corn germ portion, but also its role in activating LOX in the meal matrix.

b. Effect of linoleic acid on the aflatoxin production

During the first few days, no sporulation was observed and production of AFB_1 in linoleic-treated whole corn samples was significantly lower than the positive control. For linoleic-treated ground corn samples, the toxin synthesis was similar to the positive control (Figure 5.10 and 5.11). Ground corn may have been the better substrate since availability of all the nutrients in whole kernels may be limited. It could alsohave been due to improper distribution of the linoleic acid treatment. In the later part of the storage trial, the production of aflatoxin after showing a linear increase for two weeks remained at the same level or resulted in a gradual degradation of aflatoxin regardless of treatments or the controls. However, these changes were not significantly different from the controls. Although the inhibition of aflatoxin production was not as effective as in the phytic acid-treated sample, the inhibition in whole kernels and ground corn was about 50% and 40% respectively (Appendixes G and H).

This inhibition can be attributed to the other intrinsic components of corn, which in concert with extrinsic linoleic acid reduced the ability of *A. flavus* to synthesize aflatoxins. Furthermore, the added amount to the corn might not have been sufficient enough to completely inactivate the process of aflatoxin synthesis.

The most thorough investigations of seed pathogenesis have been conducted with maize kernels in which *Aspergillus* spp. preferentially colonize the lipid rich embryo and aleurone tissues (Burrow et al., 1997). Lipases seem to be important, since detailed histological work has demonstrated that *A. flavus*

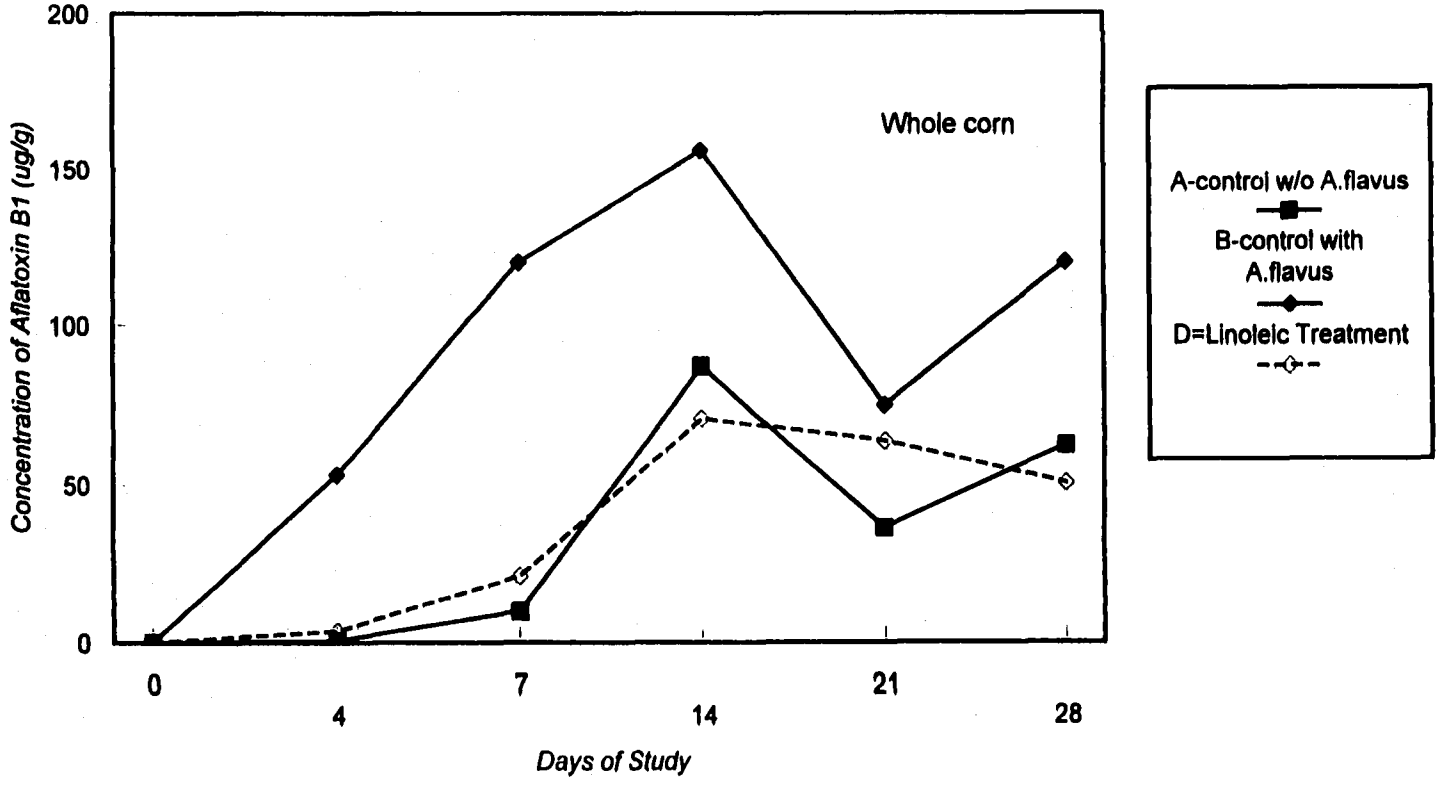

Figure 5.10 Effect of linoleic acid on the production of aflatoxin B_1 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

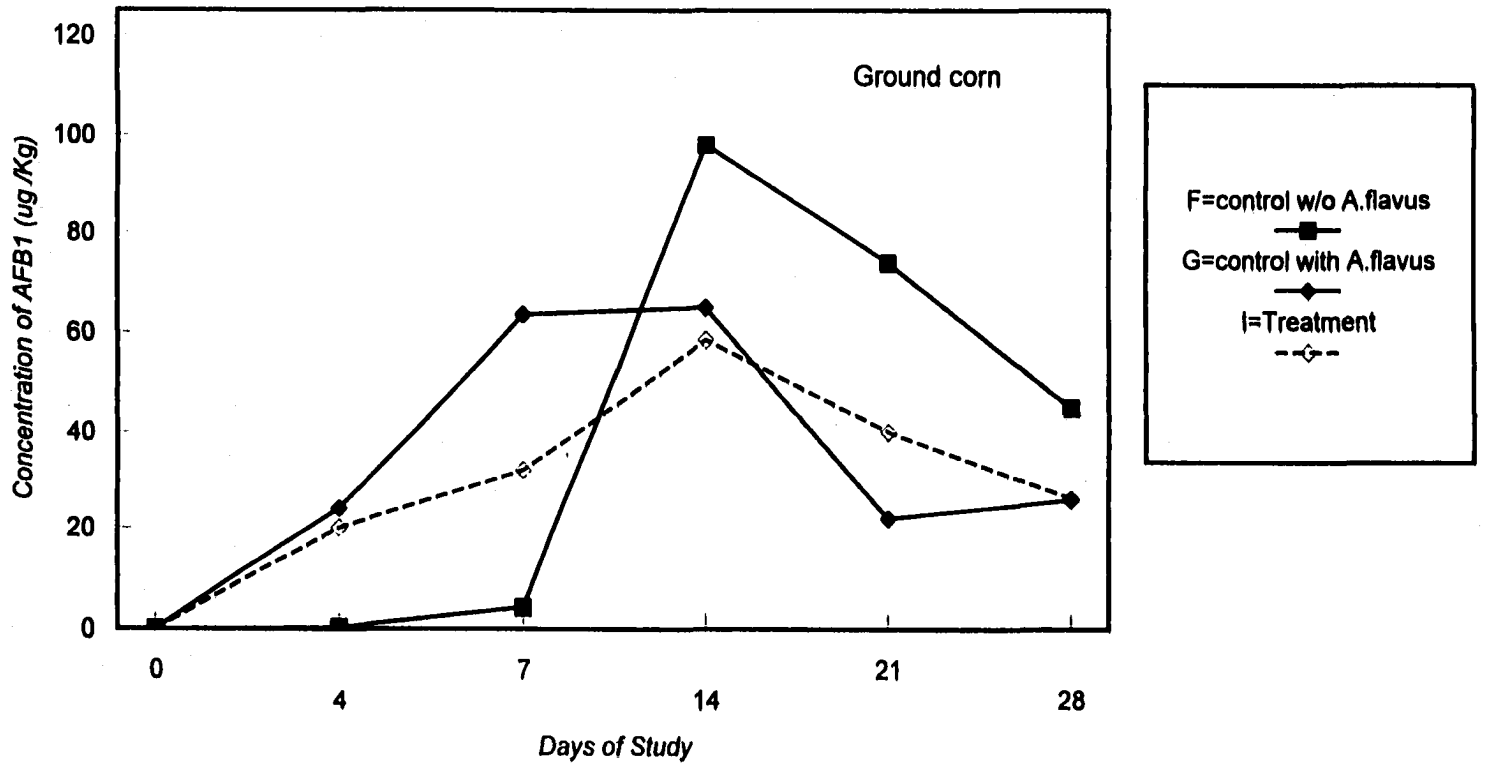

Figure 5.11 Effect of linoleic acid on the production of aflatoxin B_1 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

first destroys the lipid bodies, but not the starch granules of maize scutellum. Generally, seed lipid bodies are primarily composed of palmitic (16:0), oleic (18:1) and linoleic (18:2) acids (Burrow *et al.*, 1997). The polyunsaturated fatty acids (linoleic, and linolenic, 18:3) are subject both to enzymatic and chemical oxidation (Gardner, 1989), and lipoxygenases play an important role in the oxidation process. By considering the fact that these polyunsaturated fatty acids are a dominant part of corn and that linoleic acid was previously identified as an antimutagenic factor in corn (Burgos-Hernandez, 1998) and that it has been a part of the starting substrate, phosphotidylinositol, for the biosynthesis of phytic acid in corn, this study was conducted to determine whether linoleic acid is an important component of the *Aspergillus*-aflatoxin interaction.

Linoleic and linolenic acids are oxidized by plant stress response lipoxygenase enzymes (LOX) and result in the formation of hydroperoxy fatty acids in a stereo-specific manner to yield 9S-hydroperoxy-trans-10, cis-12-octadecadienoic acid (9S-HPODE) and or 13S-hydroperoxy-cis-9, trans-11-octadecasdienoic acid (13S-HPODE) from linolenic acid and 9S hydroperoxy-trans-10, cis-12-octadecatrienoic acid (9S-HPOTE) and/or 13S-hydroperoxy-cis-9, trans-11-octadecatrienoic acid (13S-HPOTE) from linoleic acid (Gardner, 1989). Some seed LOX-1 primarily produce 13S-HPODE and 13S-HPOTE, e.g., soybean LOX-1 (Hamberg and Samuelsson, 1967), and some produce primarily 9S-HPODE, e.g., maize embryo LOX (Gardner and Wiesleder, 1970). 13S-HPODE and 13S-HPOTE can be further metabolized to yield aldehydes with reported *in vitro* fungitoxic properties (Garner, 1991) and jasmonates which

are postulated to elicit production of plant defense proteins and secondary metabolites. A possible role of plant LOX metabolites, *Aspergillus* growth and aflatoxin production is of interest because it suggests that a natural mechanism might exist that would affect the biosynthesis of aflatoxin through linoleic acid. Both inhibition and stimulation of aflatoxin by various LOX pathway metabolites have been reported. During oxidation of these fatty acids by lipoxygenases, free fatty acids and other primary metabolites are produced. These free fatty acids and metabolites coupled with phytic acid can inactivate the metalloenzyme required activation process of aflatoxin biosynthesis through chelation. The primary products, 13S-HPODE and 13S-HPOTE and their respective aldehydes isolated from soybean were reported to inhibit germination of *A. flavus* spores and/or aflatoxin biosynthesis in maize kernels (Zeringue *et al.*, 1996, 1997). Antibacterial activity of lipid-derived volatiles, including cis-3-hexenol trans-3-hexenal, which arise from the 13-hydroperoxide of linolenic acid, has been reported (Croft *et al.*, 1993). These findings suggest a positive role afforded by linoleic acid in the antifungal properties. On the other hand, the role played by the cytochrome P450 enzyme system (CYP1A2, 2A3, 2B7, 3A3 and 3A4) in the activation of AFB1 has been addressed extensively (Roy and Kulkarni, 1997). However, an increasing number of reports have also documented that, in general, polyunsaturated fatty acids cause a marked reduction in the rate of NADPH-dependent cytochrome P450 catalyzed oxidation process in various liver preparations. More specifically, both linoleic and linolenic acid have been reported to inhibit AFB_1 activation (Burgos-

Hernandez, 1998; Firozi *et al.*, 1986; Schultz and Lueducke, 1977) by effecting NADPH mediated processes.

Although the present data do not suggest a complete inhibition in the production of aflatoxin, lower amounts of linoleic acid (1g/50mg) added to the treatment should be considered. More studies are warranted to find the optimum level of linoleic acid to counteract the biosynthesis process. Furthermore, the role of any LOX mediated activation process should also be explored.

Data from the AFB_2 trial showed a similar pattern in the production of AFB_2 in linoleic-treated samples irrespective of the type of substrate (Figures 5.12 and 5.13) which was observed in phytic acid treatments. No significant difference was found between *Aspergillus*-positive control and linoleic-treated whole corn samples. However, the production of AFB_2 in linoleic-treated ground samples was not significantly lower than that of *Aspergillus*-dependent positive controls. No significant degradation was evident in the treatments after day 14, as reported previously (Lopez-Garcia, 1998). The next section will further elucidate the possible interactions between phytic acid and linoleic acid in the same meal matrix.

c. Effect of phytic/linoleic acid combination on aflatoxin production

The data of this trial is presented in Appendixes I and J. A linear production of aflatoxins was observed in the treatments throughout the study regardless of substrate type (Figures 5.14 and 5.15). However, in ground corn after 4 days of inoculation the amount of AFB_1 remained at the same level and later started to decline after the 14^{th} day.

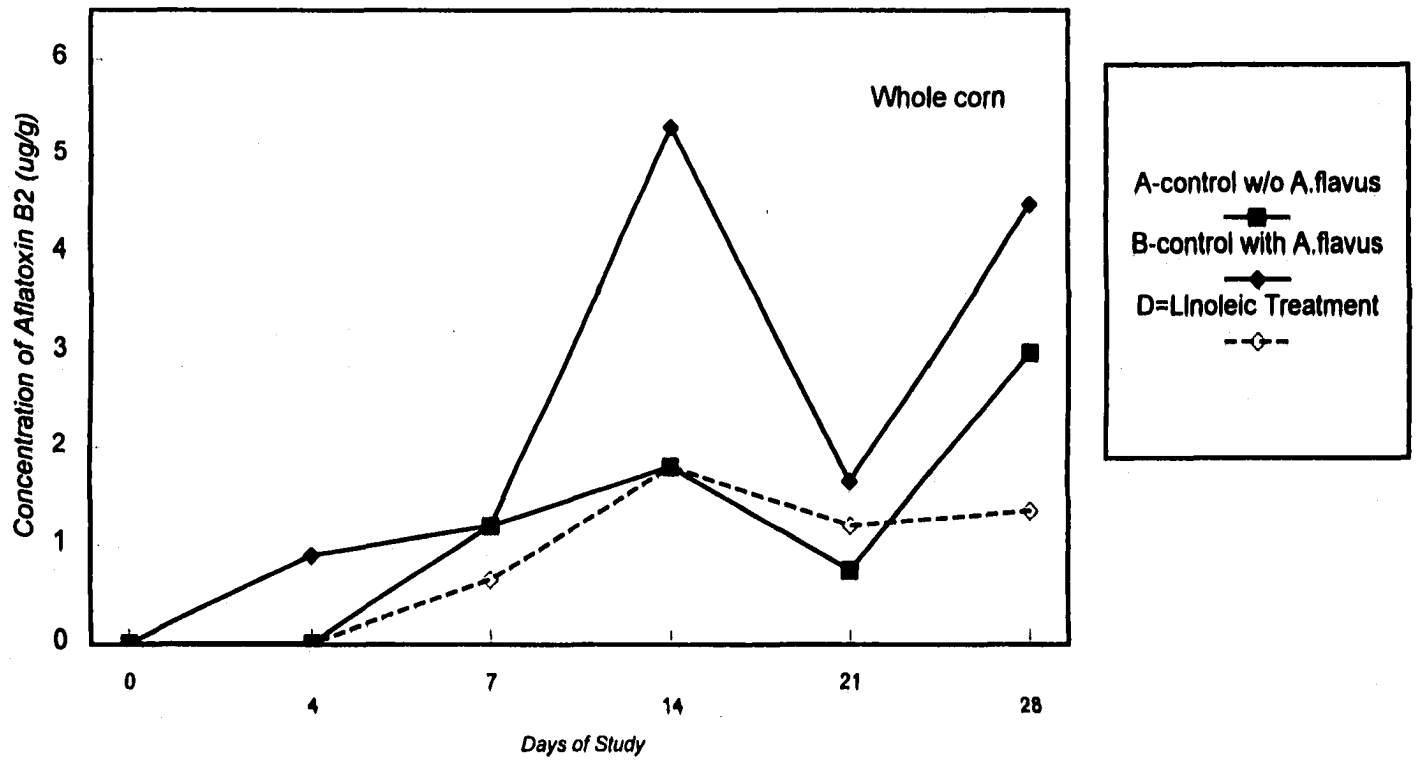

Figure 5.12 Effect of linoleic acid on the production of aflatoxin B_2 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

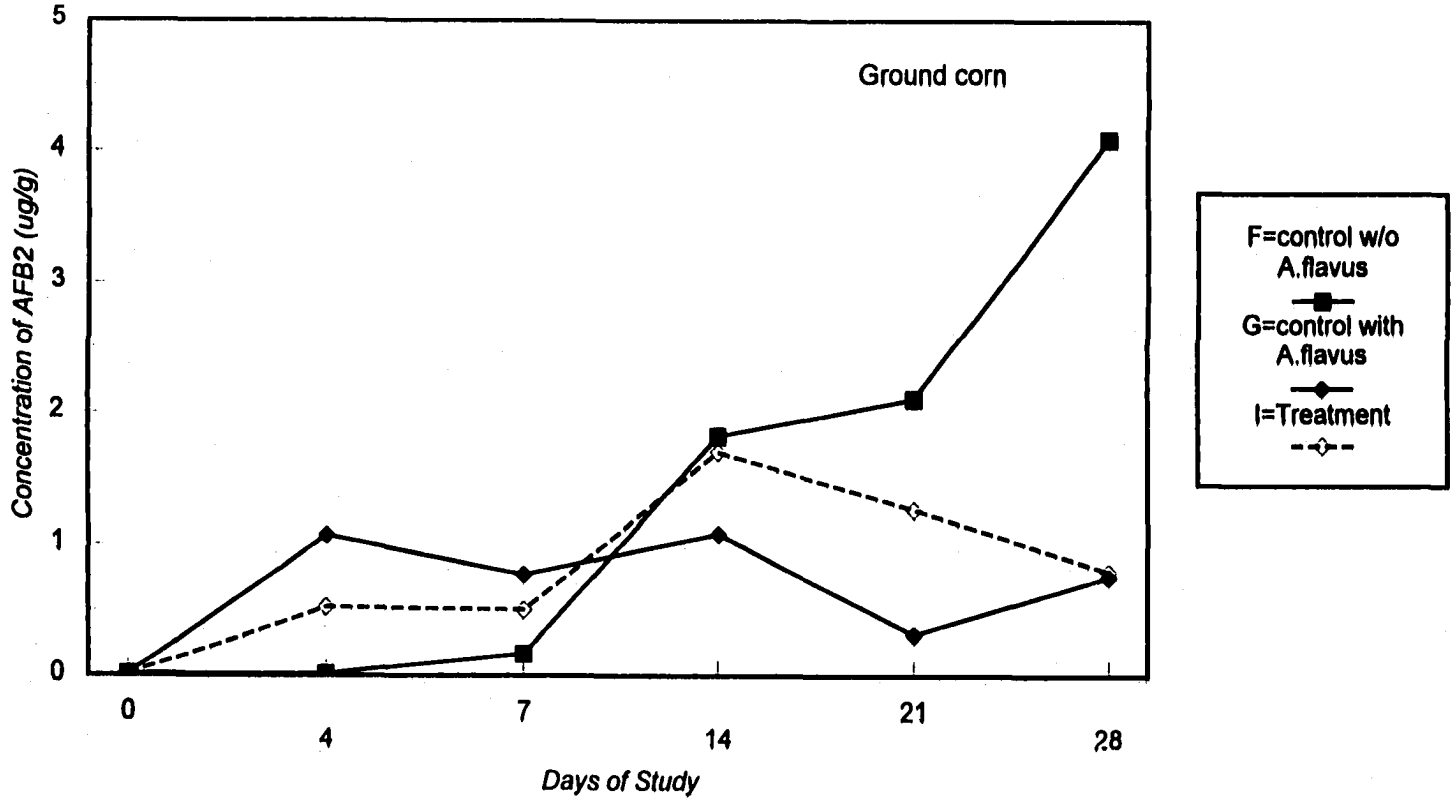

Figure 5.13 Effect of linoleic acid on the production of aflatoxin B_2 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

The production of AFB_1 in ground corn was found significantly ($p<0.05$) lower than whole corn (Figures 5.14 and 5.15). Since corn contains numerous identified and unidentified components in the matrix and there is a possibility that their intrinsic properties and exposure to added linoleic and phytic acid could have resulted in the partial degradation of AFB_1 in ground corn compared to whole corn. However, these results are contrary to the hypothesis. The hypothesis for this trial was based on the fact that by coating the outer layer of intact corn with both linoleic and phytic acid together would result in the prolonged shelf life of corn by synergistic action of these compounds in the inhibition of fungal invasion.

A similar pattern was observed in the biosynthesis of AFB2 (Figures 5.16 and 5.17. The production of aflatoxin was significantly lower in ground corn than that of whole corn.

As reported previously, aflatoxin biosynthesis is related to mold lipid biosynthesis (Townsend *et al.*, 1984). The most thorough investigations of the seed pathogenesis have been conducted with maize kernels. *Aspergillus* spp typically gain access to the seed through cracks generated by the environmental stress (heat and/or drought) or via insect damage. *Aspergillus* spp. preferentially colonize the lipid-rich embryo (Brown *et al.*, 1993) and the aleurone tissues (Keller *et al.*, 1994a). Several studies have indicated a role for specific fungal degradative enzymes (Brown *et al.*, 1993; Burow *et al.*, 1997; Cotty *et al.*, 1990), proteinous structures (Huang *et al.*, 1997) in both *Aspergillus* pathogenesis and aflatoxin biosynthesis. Furthermore, in the *Salmonella*

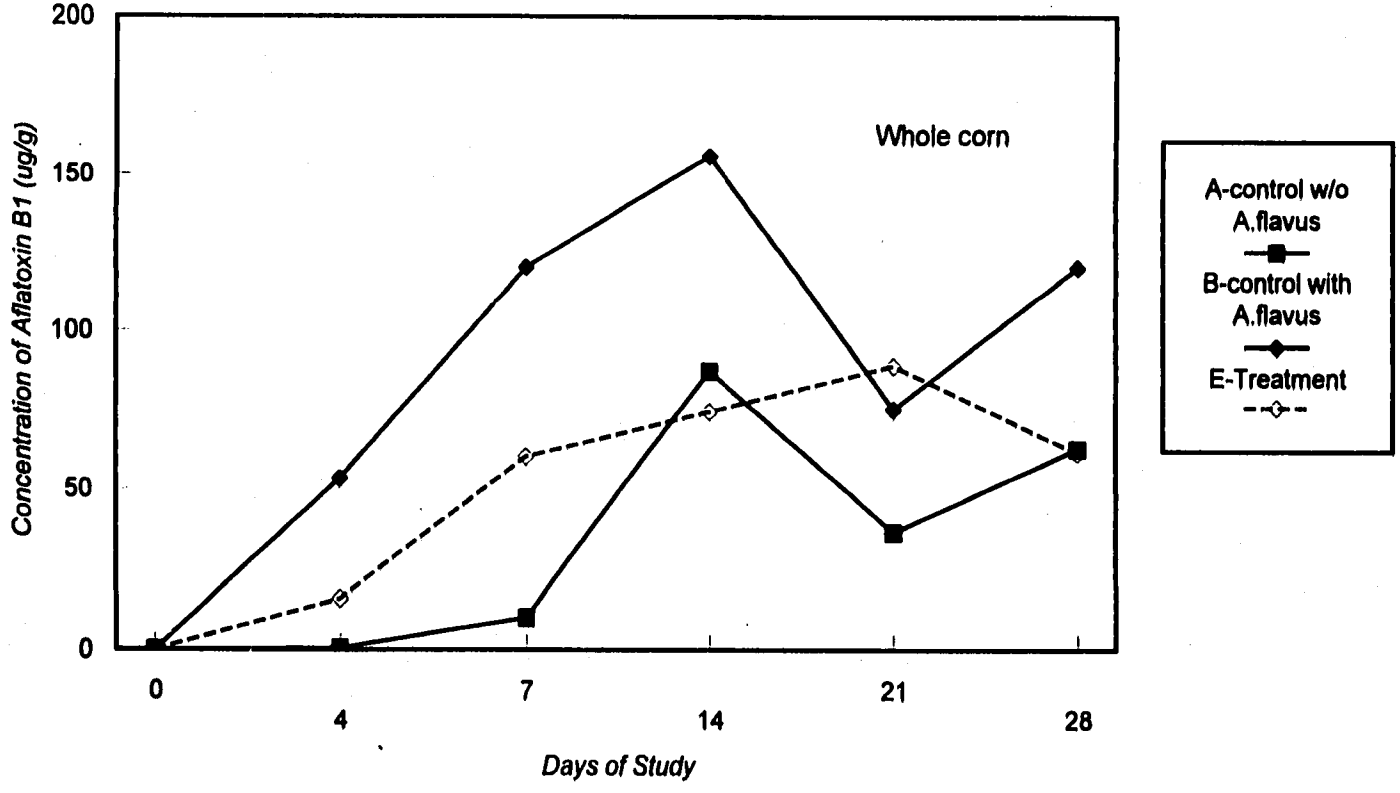

Figure 5.14 Effect of phytic/linoleic acid combination on the production of aflatoxin B_1 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

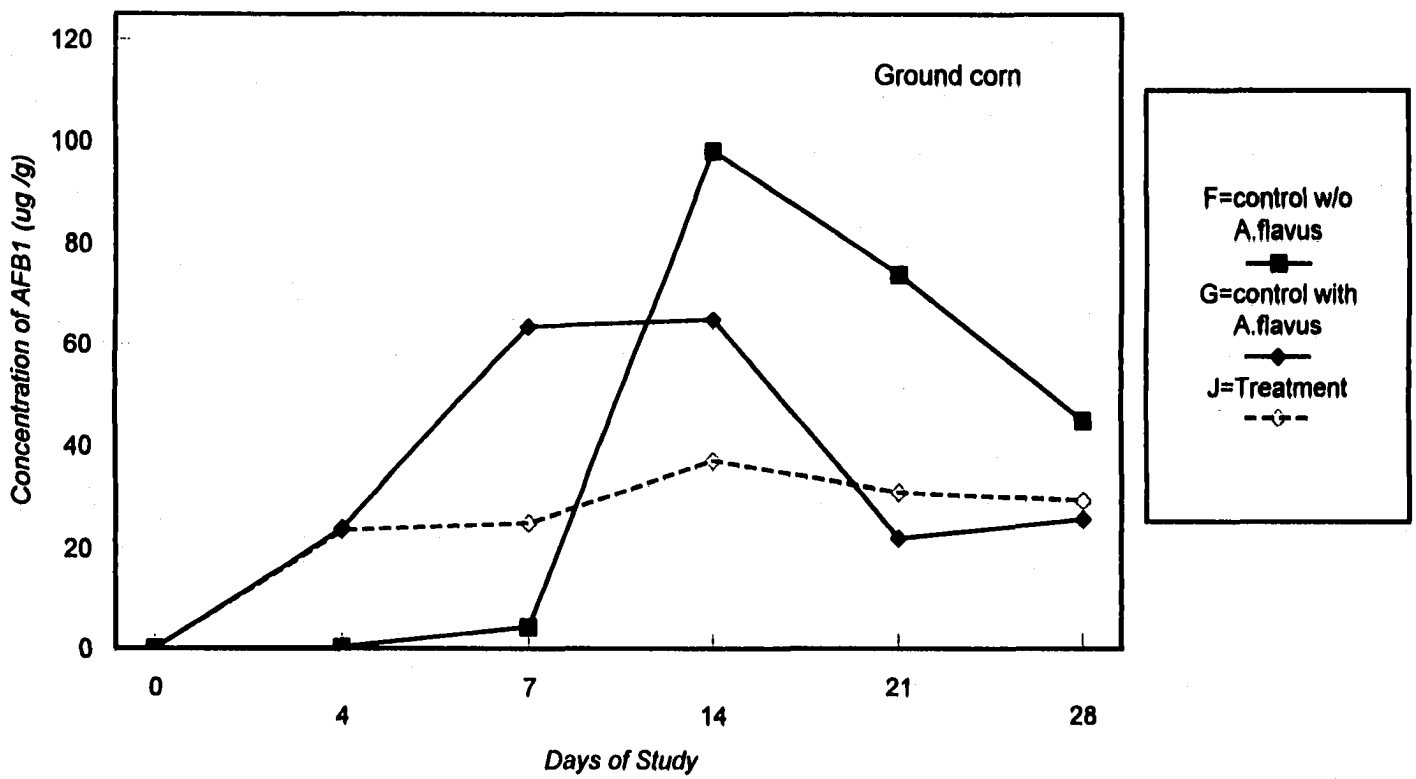

Figure 5.15 Effect of phytic/linoleic acid combination on the production of aflatoxin B_1 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

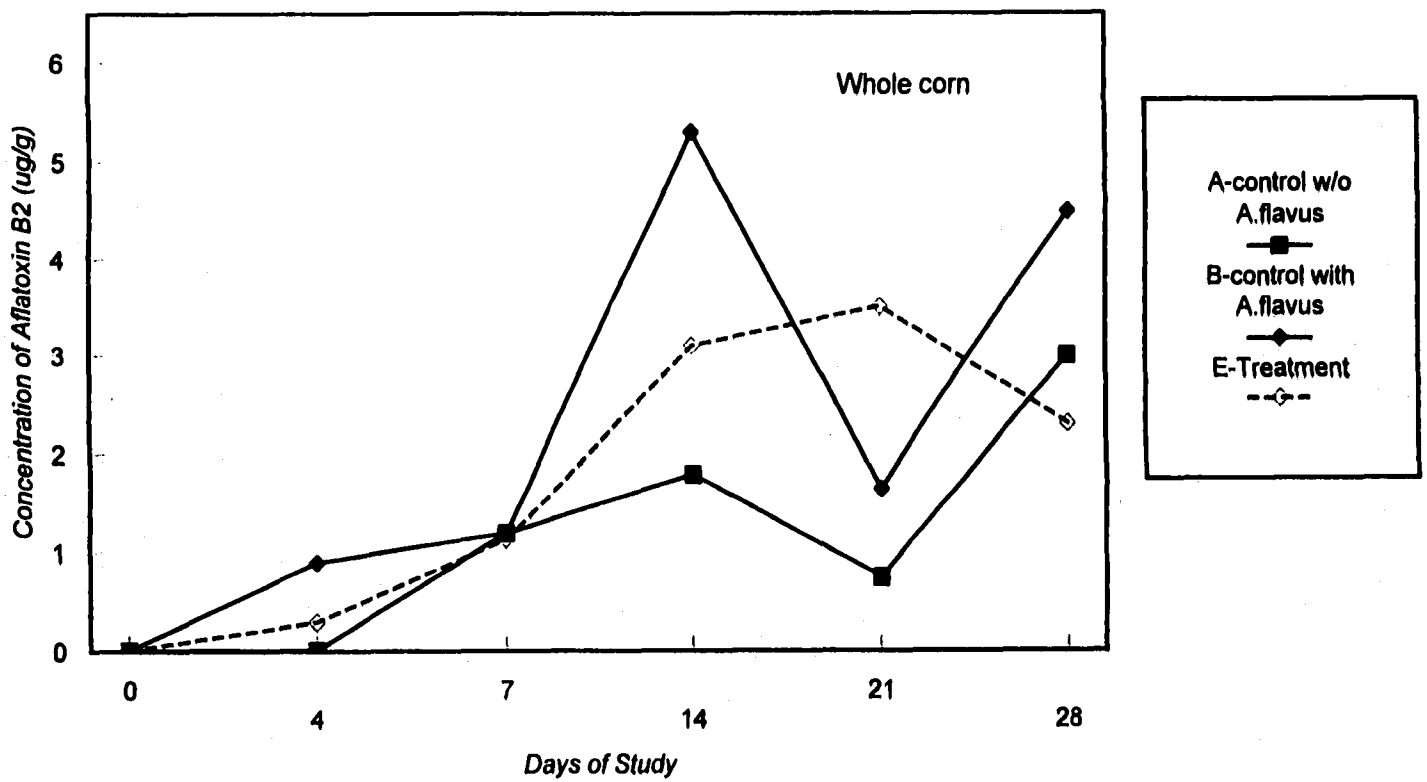

Figure 5.16 Effect of phytic/linoleic acid combination on the production of aflatoxin B_2 in whole corn kernels with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

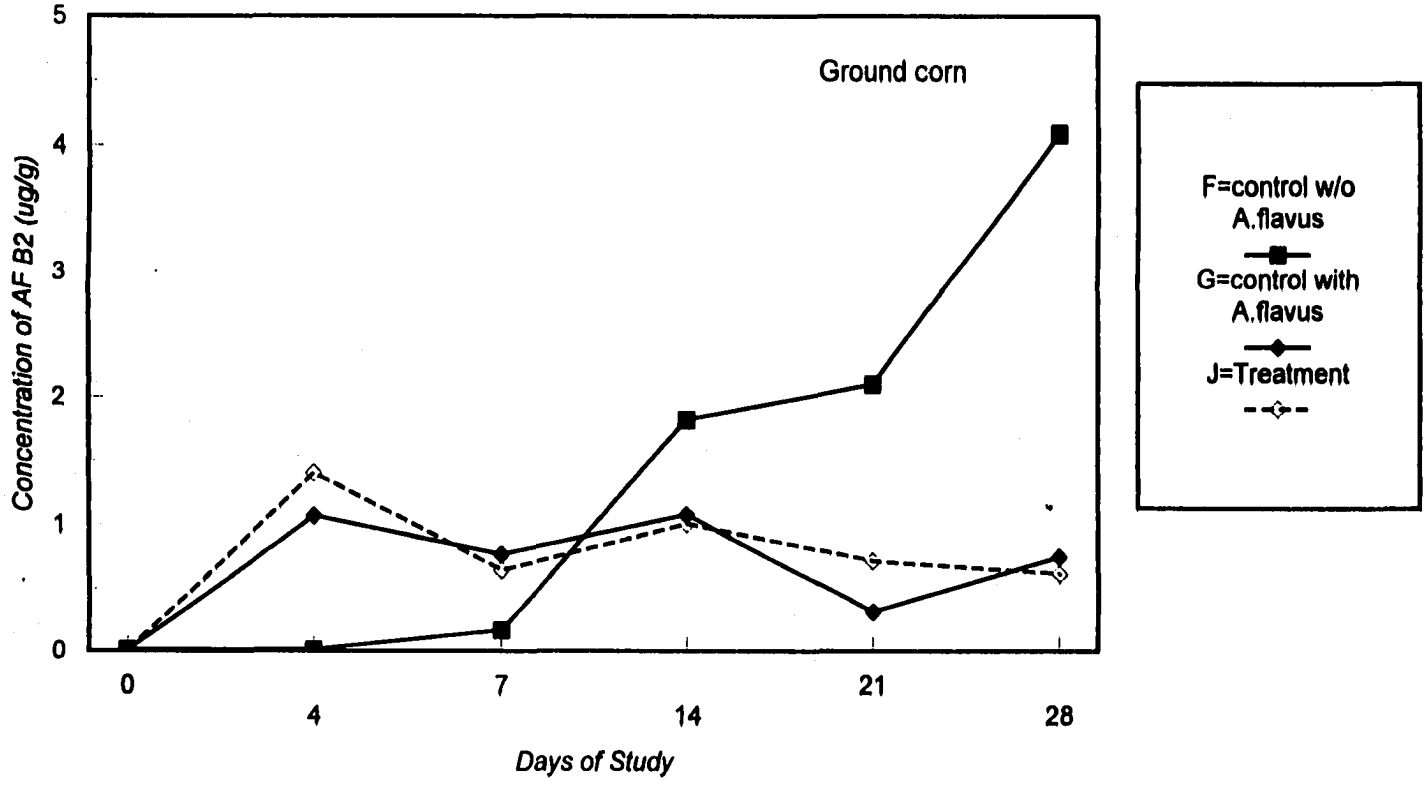

Figure 5.17 Effect of phytic/linoleic acid combination on the production of aflatoxin B_2 in ground corn with *Aspergillus flavus* inoculation. Values are mean +/- standard error of three replications.

mutagenicity assay, the low mutagenicity of higher amount of AFB_1 extracted from the corn suggested a role of these factors in the reduction of mutagenicity (Weng *et al.*, 1997). However, in the current study the combined treatment of corn by phytic acid with linoleic acid does not suggest any synergistic anti-mutagenic role in the biosynthesis of aflatoxins. But it is important to consider that the growth conditions provided to the mold were maximum (a perfect temperature and humid environment) and that the amount of phytic acid and linoleic acid were not sufficient to inhibit the process of aflatoxin biosynthesis completely, however, the data show a partial inhibition in such perfect conditions. More experiments with increased amount of these compounds are recommended to monitor *A. flavus* growth and aflatoxin biosynthesis.

Moreover, it is important to mention that the cereal grains are stored without a prior sterilization, and which is contrary to the methods adopted in this study. It is therefore recommended that more storage experiments, without autoclaving the grains, should be conducted in the presence and absence of phytic and linoleic acids.

Furthermore, it has been reported previously that by virtue of binding to proteins, phytic acid inhibits polyphenol oxidase (Graf *et al.*, 1987), α-amylase (Thompson, 1994), alcohol dehdrogenase (Altschuler and Schwartz, 1984), trypsin (Singh and Krikorian, 1982), and other enzymes. More studies are also warranted to explore its role in the activation and/or inactivation of those enzymes which takes part in the biosynthesis process of aflatoxins.

Phytic acid also has a high affinity for 2, 3-diphosphoglycerate sites in hemoglobin (Eaton and Graf, 1990), and results in the modification of heme iron-O_2 interaction, which facilitates dissociation of oxygen from hemoglobin. Therefore, phytic acid may prove useful in the treatment of ischemia, hemolytic anemia, pulmonary insufficiency and hypererythropoiesis by improving O_2 transport capabilities, if it is incorporated in erythrocytes (Nicolau *et al.*, 1986; Weiner and Franco, 1986). It is also recommended to conduct *in vivo* studies in this regard to elucidiate the mechanism of action.

6. SUMMARY AND CONCLUSIONS

Aflatoxins are naturally produced in the secondary metabolism of fungi and their presence in feed and food crops cannot be completely avoided. Aflatoxin B_1 (AFB_1), the most potent of mycotoxins, causes primary liver cancer (PLC) through necrosis, immune-suppression, gastrointestinal tract dysfunction, and pulmonary edema in animals and humans. It is metabolized by the phase I enzyme system resulting in the production of highly reactive epoxides, which cause damage to cells by covalently binding to the proteins and DNA.

Several methodologies, based on physical, chemical and biological principles, have been developed for the decontamination of aflatoxin-affected crops. Among the chemical decontamination procedures ammoniation has been demonstrated as one of the most promising measures to counteract this problem. However, the search for other means to overcome aflatoxin-ridden crops is omnipresent. Should contamination occur, then other ways to reduce the health risks posed by these metabolites must be adopted. Recent research has focused on identifying naturally-occurring components in plants to deal with potential hazards. Plants contain innumerable potential antimutagenic compounds. Currently, the role of plant-derived dietary fibers in the protection of human mutagenesis has been extensively studied. The major dietary fibers derive from cereal grains and contain several such components in its intricacy. Various studies *in vivo* and *in vitro* have shown the anti-mutagenic and anti-fungal potential of phytic acid. Besides phytic acid, these fibrous portions of plants also contain anti-oxidative fatty acids and vitamins.

.The first objective of the current study was to evaluate an interaction between commercially available phytic acid or phytate (inositol hexaphosphate) and pure aflatoxin B_1. During the course of this study antimutagenic properties of phytic acid, linoleic acid and phosphotidylinositol against indirect-acting (Aflatoxin B_1 [AFB_1] & 2 aminofluorene [2-AF]) and direct-acting (sodium azide [NaN_3] & methyl nitro-*N*- nitrosoguanidine [MNNG]) in the Ames *Salmonella* microsomal mutagenicity assay were studied. The standard plate incorporation procedure of Ames assay was used employing tester strains TA-100 and TA-98. The mutagenic activity of the carcinogens was tested in the presence of the various salts of phytic acid, linoleic acid, and phosphotidylinositol at different concentrations.

A reduction in the number of revertants in the *Salmonella*/microsomal mutagenicity assay was shown to be a function of increased concentration of phytic acid. Na-, Ca-, Mg-, & K-salts of phytic acid and *myo*-inositol substantially reduced the number of revertants irrespective of mutagen type. The largest reduction (20-50%) was observed against direct-acting NaN_3, i.e., Ca-salt (10-55%), Na-salt (10-20%) and *myo*-inositol (20-35%). Against AFB_1, the reduction was: Ca-salt (20%), K-salt (20-50%), Mg-salt (10-30%), and *myo*-inositol (20-40%). Similar results were observed with MNNG and 2-AF in the same assay. However, the results of the phytic acid trial did not suggest it was a complete anti-mutagenic compound in the Ames test. However, this was the first study regarding the evaluation of phytic acid and its various salts in the *Salmonella*/microsomal mutagenicity assay. Further investigations on the role

of phytic acid are warranted *in vivo* to evaluate its potential anti-mutagenic properties against aflatoxin B_1 mutagenicity.

On the other hand, linoleic acid and phosphotidylinositol showed antimutagenic potentials in this assay against most of the mutagens with both tester strains (TA-98 and TA-100). Linoleic acid has been previously shown to be anti-mutagenic both *in vivo* and *in vitro*. The potential role for this compound in mutagenesis should also be investigated against indirect-acting aflatoxin B_1 *in vivo*.

Although phytic acid (various salts) did not render a complete antimutagenic potential in the Ames *Salmonella/* microsomal mutagenicity assay against aflatoxin B_1, it did, however, show its antimutagenicity against another indirect-acting mutagen, i.e., 2-aminofluorene, and direct-acting sodium azide and methyl-N-nitroso-nitrosoguanidine. Linoleic acid, a component of phosphotidylinositol for phytic acid phosphorylation was anti-mutagenic. These results suggest that if phytic acid and linoleic acid were made available in the diet through the introduction of more fibrous portions of cereals, it could provide protection against carcinogenesis.

Phytic acid is found in most cereals and has been implicated as anti-nutritive due to its chelating properties with divalent ions. It has been reported that trace elements in most cereals occur in the aleurone layer (Lee *et al.*, 1966; Lillihoj *et al.*, 1974) and support the growth of *Aspergillus* with the exception of corn where they occur predominantly in the germ portion (Burow *et al.*, 1997; Zhou and Erdman, 1995).

The second objective of this study was to evaluate the anti-mutagenic potential of phytic acid in a synthetic liquid medium and the factors that govern its chelating properties on the biosynthesis of aflatoxins by *Aspergillus flavus*. This study was conducted to evaluate the efficacy of phytic acid on the production of AFB_1 by *Aspergillus flavus* in the Czapek-Dox liquid medium.

It was evident from the results of this study that as the amount of phytic acid was increased, the production of AFB_1 gradually decreased and was completely inhibited by the amount > 0.5mg phytic acid/100ml liquid medium. It has been reported previously that metal ions are important for optimal growth of fungi and chelators may affect the production of AFB_1 by altering the growth conditions of the fungi. To confirm whether metal ions played any role in the biosynthesis process of aflatoxins, a second experiment was conducted to study the effects of different metal ions absent in the medium. According to the results, in the absence of $FeSO_4$ and $ZnSO_4$, the production of AFB_1 was completely inhibited and the production of aflatoxin was found significantly lower than *Aspergillus*-dependent controls. However, the absence of $CuSO_4$ and $MgSO_4$ did not exhibit the same response, but the synthesis of aflatoxin was lower than that of the controls.

On the basis of these results it has been suggested that when seeds of plants are sprayed or soaked with phytic acid solution with the appropriate concentration prior to storage, protection from mold invasion can be achieved. Subsequently, their treatment could also reduce the incidence of cancer by inhibiting the biosynthesis of aflatoxins. However, this approach will be very

cumbersome and expensive. Therefore, it is recommended to further elucidate the mechanism of action of phytic acid on the inhibition of aflatoxin biosynthesis. It will be more plausible to develop such varieties of the grain that could contain either more phytic acid or the enzymes required for its biosynthesis in plant physiological conditions.

The next phase of this study was designed to evaluate the effects of these factors in clean corn samples where indigenous activity of phytic acid and other components may have the same effect on aflatoxin biosynthesis.

The third and last objective of this study was to evaluate the role of phytic acid and its other counterparts, i.e., linoleic acid, towards the production of aflatoxins during corn storage. It was hypothesized on the basis of previous trials that the presence of phytic acid along with other anti-mutagenic factors such as linoleic acid, which is part of the initial substrate for phytic acid formation, the phosphotidylinositol in corn would affect the formation of aflatoxins through its antifungal and/or antioxidant potential in the long-term storage of corn.

Experimental data confirmed that molds can degrade their own metabolites after a certain period of growth as reported previously. Although not a complete inhibition was achieved in corn samples regardless of whole kernels or ground type, a significant inhibition was observed in the ground corn compared to whole kernels. Corn contains a variety of antimutagenic components i.e., minerals, fatty acids, proteinous compounds, inside its outer layer, therefore it is likely that the inhibition of aflatoxin synthesis would have

been more in the ground corn due to the exposure of all of these components to the mold. The chelation of metal ions by phytic acid has been reported extensively in the literature and in the liquid medium trial of current study. Binding of phytic acid to zinc or iron is believed to result in the low production of aflatoxin compared to *Aspergillus*-control treatments in soybean; hence, decreased yields of aflatoxin in phytic acid treatments should have resulted from an increased amount of phytic acid. It is therefore likely that a direct exposure of the mineral ions i.e., iron and zinc, would have resulted in the comparable reduction of aflatoxin biosynthesis in ground corn compared to whole kernels. More studies are warranted, however, to evaluate any other metabolite produced during this process.

Furthermore, the results of this trial did not confirm the levels of phytic acid required to inhibit the process of aflatoxin biosyntheis. It is important to consider that only 1g of phytic acid was added to both whole and ground treatments. More studies are recommended to evaluate the dose-dependent process of phytic acid's anti-fungal properties.

In the case of linoleic-treated corn samples, although the present results did not show an inhibition similar to phytic acid-treated corn, the data indicated a significantly lower amount of aflatoxins. However, once again the lower amounts of linoleic acid (1g/50g) added to the treatment should be considered. More studies are warranted to find the optimum level of linoleic acid to counteract the biosynthesis process. Although the objectives of this study were not to evaluate the process of sporulation and the factors that govern their

growth, it has been reported elsewhere in the literature that lipoxygenases are activated by unsaturated fatty acids and subsequently activate biosynthesis of aflatoxins through *Aspergilli* sporulation. Further research on the role of lipoxygenase-mediated activation process should also be explored. Protein contents have also been reported as inhibiting factors in aflatoxin biosynthesis in maize (Prasad *et al.*, 1996); hence, the role of protein contents besides phytic acid in inactivating a biosynthesis pathway should be further explored.

Furthermore, it has been reported previously that by virtue of binding to proteins, phytic acid inhibits polyphenol oxidase (Graf *et al.*, 1987), α-amylase (Thompson, 1994), alcohol dehdrogenase (Altschuler and Schwartz, 1984), trypsin (Singh and Krikorian, 1982), and other enzymes. More studies are also warranted to explore its role in the activation and/or inactivation of those enzymes which takes part in the biosynthesis process of aflatoxins.

Based upon the results of these experiments, it is recommended that phytic acid and linoleic acid should be investigated from a pragmatic viewpoint and their role in different stages of plant growth and during storage should be investigated. Furthermore, investigations regarding their anti-carcinogenic properties, especially in the case of the less investigated phytic acid, against aflatoxins *in vivo* are also warranted.

Finally, the development of genetically-altered varieties of corn with a higher amount of phytic acid could ultimately be a solution to overcome mold invasion.

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APPENDIX A EFFECT OF PHYTIC ACID CONCENTRATION ON PRODUCTION OF AFB_1 IN CZAPEK-DOX LIQUID MEDIUM

Days of Incubation		Culture Number/Treatments A[b]	B[b]	C[b]	D[b]	E[b]	F[b]	G[b]
Phytic Acid (mg/100ml)		**0**	**0.01**	**0.05**	**0.10**	**0.20**	**0.50**	**1**
AFB1 (ug/100ml)	**5**	8.50 ±0.05	4.00 ±1.1	1.75 ±0.1	1.75 ±0.1	0.55[a] ±0.02	<0.1[a] ±0.01	<0.10[a] ±0.01
	10	9.50 ±0.20	8.50 ±0.50	2.70 ±0.2	1.60 ±0.10	1.00 ±0.50	<0.10[a] ±0.01	<0.10[a] ±0.01
	15	12.90 ±1.80	12.00 ±1.50	4.25 ±0.80	7.90 ±0.10	0.95[a] ±0.02	<0.10[a] ±0.01	<0.10[a] ±0.01
	25	27.00 ±2.30	23.50 ±3.30	10.50 ±0.22	4.80 ±0.50	1.85[a] ±0.07	<0.10[a] ±0.01	<0.10[a] ±0.01

a = Significant at $p<0.05$
b = All treatments are mean ± std. error of three replications

APPENDIX B EFFECT OF METAL IONS ON THE PRODUCTION OF AFB_1 FROM *A. FLAVUS*

Days of Incubation		Culture Number/Treatments A[a]	B[a]	C[a]	D[a]	E[a]	F[a]	G[a]
Phytic Acid (mg/100ml)		**0**	**0.1**	**1**	**0.1**	**0.1**	**0.1**	**0.1**
AFB1 (ug/100ml)	**5**	10.70 ±0.50[a]	2.60 ±0.20	<0.10 ±0.05	<0.10 ±0.01	<0.10 ±0.01	<0.10 ±0.15	<0.10 ±0.05
	10	12.60 ±2.50	3.40 ±1.50	<0.10 ±0.05	4.50 ±0.86	<0.10 ±0.01	1.40 ±1.00	<0.10 ±0.05
	15	20.50 ±2.60	5.90 ±1.00	<0.10 ±0.05	17.00 ±1.10	<0.10 ±0.01	2.60 0.50	<0.10 ±0.05

a = All treatments are mean ± std. error of three replications
D=without $CuSO_4$ E=without $FeSO_4$
F=without $MgSO_4$ G=without $ZnSO_4$

APPENDIX C AFB_1 (ng/g) PRODUCTION IN PHYTIC ACID TREATED WHOLE CORN

Tmt	Number of days				
	4	7	14	21	28
A	$5.06x10^2$ $\pm 9.0x10^2$	$9.81x10^3$ $\pm 2.8x10^3$	$8.70x10^4$ $\pm 2.3x10^4$	$1.62x10^4$ $\pm 5.5x10^3$	$6.20x10^4$ $\pm 1.6x10^4$
B	$5.30x10^4$ $\pm 3.7x10^4$	$1.24x10^5$ $\pm 4.5x10^3$	$1.55x10^5$ $\pm 1.1x10^4$	$7.20x10^4$ $\pm 1.9x10^4$	$1.29x10^5$ $\pm 3.4x10^4$
C	$1.50x10^2$ $\pm 4.4x10^1$	$3.50x10^3$ $\pm 2.8x10^3$	$4.38x10^4$ $\pm 1.1x10^4$	$3.35x10^4$ $\pm 1.2x10^4$	$3.24x10^4$ $\pm 4.2x10^3$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
A = Corn w/o *A. flavus*; B = Corn with *A. flavus* C = Corn with *AF* + Tmt

Values are mean ± standard error of three replications.

APPENDIX D AFB_1 (ng/g) PRODUCTION IN PHYTIC ACID TREATED GROUND CORN

Tmt	Number of days				
	4	7	14	21	28
F	$3.06x10^2$ ± 93	$4.27x10^3$ ± $1.2x10^3$	$9.77x10^4$ ± $8.9x10^2$	$7.39x10^4$ ± $3.7x10^4$	$9.95x10^4$ ± $7.6x10^4$
G	$2.25x10^4$ ± $7.7x10^3$	$5.29x10^4$ ± $4.2x10^4$	$6.47x10^4$ ± $2.6x10^4$	$2.19x10^4$ ± $1.1x10^4$	$2.59x10^4$ ± $1x10^4$
H	$7.90x10^2$ ± $2.7x10^2$	$5.94x10^3$ ± $1.9x10^3$	$1.02x10^4$ ± $6.5x10^3$	$4.21x10^3$ ± $6.4x10^2$	$3.63x10^3$ ± $1.7x10^3$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
F = Corn w/o *A. flavus*; G = Corn with *A. flavus*; H = Corn with *AF* + Tmt
Values are mean± standard error of three replications.

APPENDIX E AFB_2 (ng/g) PRODUCTION IN WHOLE CORN

Tmt	Number of days				
	4	7	14	21	28
A	1.33 ± 0.57	1.33 ± 0.57	$9.17x10^2$ ± $4.1x10^2$	$5.0x10^2$ ± $3.7x10^2$	$3.05x10^2$ ± $3.0x10^2$
B	1.33 ± 0.57	$6.09x10^2$ ± $4.2x10^2$	$3.47x10^3$ ± $2.6x10^2$	$1.43x10^3$ ± $1.2x10^3$	$2.92x10^3$ ± $1.0x10^4$
C	1.33 ± 0.57	$3.09x10^1$ ± $2x10^1$	$6.47x10^2$ ± $1.6x10^2$	$1.23x10^2$ ± $1.2x10^2$	$1.20x10^2$ ± $1.0x10^1$
D	$5.30x10^1$ ± $9.5x10^0$	$1.85x10^2$ ± $1.6x10^2$	$2.96x10^3$ ± $2.1x10^2$	$1.80x10^3$ ± $1.5x10^3$	$2.02x10^3$ ± $5.5x10^2$
E	$3.14x10^2$ ± $1x10^1$	$9.22x10^1$ ± $1.1x10^1$	$3.01x10^3$ ± $1.5x10^3$	$3.54x10^3$ ± $1.8x10^2$	$2.34x10^3$ ± $6.5x10^2$

Amount of AFB_1 in clean corn = 1.33 ± 0.57 ng/g
A = Corn w/o *A. flavus*; B = Corn with *A. flavus*; C, D, E = Corn with *AF* + Tmt. Values are mean± standard error of three replications.

APPENDIX F AFB_2 (ng/g) PRODUCTION IN GROUND CORN

Tmt	Number of days				
	4	7	14	21	28
F	1.33 ± 0.57	4.13 ± 7.15	$1.17x10^2$ ± $1x10^2$	$1.43x10^3$ ± $1.07x10^3$	$2.63x10^3$ ± $2.6x10^3$
G	$7.25x10^2$ ± $6.2x10^2$	$2.59x10^2$ ± $4.2x10^2$	$6.47x10^2$ ± $2.6x10^2$	$2.11x10^2$ ± $1.5x10^2$	$5.13x10^2$ ± $1x10^2$
H	$6.75x10^1$ ± $6.5x10^1$	$1.06x10^2$ ± $4.5x10^1$	$1.62x10^2$ ± $1.6x10^2$	$8.3x10^1$ ± $1.5x10^1$	$5.7x10^1$ ± $0.7x10^1$
I	$7.31x10^2$ ± $2.2x10^2$	$3.36x10^2$ ± $3.54x10^2$	$1.73x10^3$ ± $8.13x10^2$	$1.30x10^3$ ± $1.7x10^2$	$8.11x10^2$ ± $5x10^2$
J	$1.41x10^3$ ± $1.17x10^3$	$6.45x10^2$ ± $2.67x10^2$	$1.39x10^3$ ± $5.5x10^2$	$7.36x10^2$ ± $1.3x10^2$	$6.71x10^2$ ± $7.3x10^1$

Amount of AFB_1 in clean corn = 1.334 ± 0.57 ng/g
F = Corn w/o *A. flavus*; G = Corn with *A. flavus*; H, I, J = Corn with *AF* + Tmt.
Values are mean± standard error of three replications.

APPENDIX G AFB_1 (ng/g) PRODUCTION IN LINOLEIC ACID TREATED WHOLE CORN

Tmt	Number of days				
	4	7	14	21	28
A	$5.06x10^2$ $\pm 9.0x10^2$	$9.81x10^3$ $\pm 2.8x10^3$	$8.70x10^4$ $\pm 2.3x10^4$	$1.62x10^4$ $\pm 5.5x10^3$	$6.20x10^4$ $\pm 1.6x10^4$
B	$5.30x10^4$ $\pm 3.7x10^4$	$1.24x10^5$ $\pm 4.5x10^3$	$1.55x10^5$ $\pm 1.1x10^4$	$7.20x10^4$ $\pm 1.9x10^4$	$1.29x10^5$ $\pm 3.4x10^4$
D	$3.54x10^3$ $\pm 9.9x10^2$	$2.21x10^4$ $\pm 1.80x10^3$	$7.12x10^4$ $\pm 5.9x10^3$	$6.80x10^4$ $\pm 8.7x10^3$	$5.08x10^4$ $\pm 1.0x10^4$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
A = Corn w/o *A. flavus*; B = Corn with *A. flavus*; D = Corn with *AF* + Tmt
Values are mean± standard error of three replications.

APPENDIX H AFB_1 (ng/g) PRODUCTION IN LINOLEIC ACID TREATED GROUND CORN

Tmt	Number of days				
	4	7	14	21	28
F	$3.06x10^2$ $\pm 9.3x10^1$	$4.27x10^3$ $\pm 1.2x10^3$	$9.77x10^4$ $\pm 8.9x10^2$	$7.39x10^4$ $\pm 3.7x10^4$	$4.95x10^4$ $\pm 1.6x10^4$
G	$2.25x10^4$ $\pm 7.7x10^3$	$5.29x10^4$ $\pm 4.2x10^4$	$6.47x10^4$ $\pm 2.6x10^4$	$2.19x10^4$ $\pm 1.1x10^4$	$2.59x10^4$ $\pm 1.0x10^4$
I	$2.28x10^4$ $\pm 9.5x10^3$	$3.61x10^4$ $\pm 2.3x10^4$	$5.96x10^4$ $\pm 2.1x10^4$	$4.10x10^4$ $\pm 4.7x10^3$	$2.71x10^3$ $\pm 1.2x10^3$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
F = Corn w/o *A. flavus*; G = Corn with *A. flavus*; I = Corn with *AF* + Tmt

Values are mean± standard error of three replications.

APPENDIX I AFB_1(ng/g) PRODUCTION IN LINOLEIC/PHYTIC ACID TREATED WHOLE CORN

Tmt	Number of days				
	4	7	14	21	28
A	$5.06x10^2$ $\pm 9.0x10^2$	$9.81x10^3$ $\pm 2.8x10^3$	$8.70x10^4$ $\pm 2.3x10^4$	$1.62x10^4$ $\pm 5.5x10^3$	$6.20x10^4$ $\pm 1.6x10^4$
B	$5.30x10^4$ $\pm 3.7x10^4$	$1.24x10^5$ $\pm 4.5x10^3$	$1.55x10^5$ $\pm 1.1x10^4$	$7.20x10^4$ $\pm 1.9x10^4$	$1.29x10^5$ $\pm 3.4x10^4$
E	$2.12x10^4$ $\pm 7.9x10^3$	$5.95x10^4$ $\pm 1.0x10^4$	$8.02x10^4$ $\pm 3.8x10^4$	$8.99x10^4$ $\pm 5.8x10^3$	$6.17x10^4$ $\pm 1.3x10^4$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
A = Corn w/o *A. flavus*; B = Corn with *A. flavus* E = Corn with *AF* + Tmt
Values are mean± standard error of three replications.

APPENDIX J AFB_1 (ng/g) PRODUCTION IN LINOLEIC/PHYTIC ACID TREATED GROUND CORN

Tmt	Number of days				
	4	7	14	21	28
F	$3.06x10^2$ $\pm 9.3x10^1$	$4.27x10^3$ $\pm 1.2x10^3$	$9.77x10^4$ $\pm 8.9x10^2$	$7.39x10^4$ $\pm 3.7x10^4$	$9.95x10^4$ $\pm 7.6x10^4$
G	$2.25x10^4$ $\pm 7.7x10^3$	$5.29x10^4$ $\pm 4.2x10^4$	$6.47x10^4$ $\pm 2.6x10^4$	$2.19x10^4$ $\pm 1.1x10^4$	$2.59x10^4$ $\pm 1.0x10^4$
J	$3.04x10^4$ $\pm 1x10^4$	$2.55x10^4$ $\pm 6.3x10^3$	$4.46x10^4$ $\pm 1.8x10^4$	$3.08x10^4$ $\pm 2.4x10^3$	$2.97x10^4$ $\pm 8.6x10^2$

Amount of AFB_1 in clean corn = 12.4 ± 1.68 ng/g
F = Corn w/o *A. flavus*; G = Corn with *A. flavus*; J = Corn with *AF* + Tmt.

Values are mean± standard error of three replications.

VITA

The author was born on January 2, 1968, the eldest child of Mr. Syed Liaquat Ali Shah and Mrs. Mairaj Liaquat. He completed his higher secondary school in 1984 from F. G. Degree College, Quetta, Pakistan. In January, 1985, the author entered University of Agriculture, Faisalabad, Pakistan, and was graduated with a bachelor of science degree, majoring in food science and Technology, in 1988.

He was later appointed as Research Officer in the Food Technology section of the Balochistan Agricultural Research Institute, Quetta, Pakistan, where he worked in collaboration with the Food and Agricultural Organization of the United Nations for two years. During his job, the author excelled in the research area of post-harvest physiology of deciduous fruits.

The author received his master of science degree on a Thomas Jefferson Fellowship from North Carolina State University in 1995. He was accepted at Louisiana State University to pursue a doctral degree in Food Science with an interdepartmental concentration in toxicology. His research interests focused on the evaluation of the anti-mutagenic potential of intrinsic components in plants against both food-borne and environmental contaminants. Currently, he is a candidate for the degree of Doctor of Philosphy, which will be conferred in December, 1999.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Syed Shahid Ali

Major Field: Food Science

Title of Dissertation: Risk Management of Aflatoxin Through Mutagenic Potential Modification and Toxin Formation by Intrinsic Components in Food

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

July 15, 1999